Variation in central serotoninergic 5-HT1B function through the light-dark cycle: effect of chronic antidepressant treatment

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Variation in central serotoninergic 5-HT_{1B} function through the light-dark cycle: Effect of chronic antidepressant treatment.

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Abstract

An abnormal serotonin (5-HT) system has been thought to underlie the aetiology of depression and drugs that target the 5-HT system are clinically effective at alleviating depression. Antidepressants can affect circadian (24 hour) rhythms, some of which are abnormal in depression. Central serotoninergic function, which displays a marked circadian rhythm, is under the control of terminal 5-HT_{1B} autoreceptors. This thesis addresses the hypothesis that prolonged antidepressant treatment differentially alters the control the 5-HT_{1B} receptor exerts on synthesis and release through the light-dark cycle.

The basal synthesis rate of 5-HT varied significantly in the hypothalamus, hippocampus, frontal cortex and striatum over the light-dark cycle. RU24969, a 5-$HT_{1A/1B}$ agonist, significantly decreased the rate of 5-HT synthesis in all brain regions. The degree of inhibition over the light-dark cycle varied significantly in the hippocampus, frontal cortex and striatum. The response was pharmacologically characterised as being mediated by a 5-HT_{1B} receptor. The variation in the RU24969-induced decrease in 5-HT synthesis was significantly altered by chronic treatment with two antidepressant drugs; desipramine a noradrenaline uptake inhibitor and paroxetine a serotonin uptake inhibitor.

In vivo microdialysis was used to investigate 5-HT_{1B} control of 5-HT release in the anterior hypothalamus of anaesthetised rats at mid light and end light. The release-suppressing effects of RU24969 were significantly greater when measured at end light compared to mid light. After chronic antidepressant treatment, as above, the effect of RU24969 was significantly attenuated only at end light.

The results agree with the proposed hypothesis, indicating that chronic antidepressant treatment affects 5-HT_{1B} control of release and synthesis in a phase-dependent manner. A new model is proposed to explain how antidepressant drugs might alleviate depression with a circadian abnormality by their interaction with 5-HT_{1B} autoreceptors.

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Contents

To Poppy and Horrid

Chapter 1 Introduction

This thesis is concerned with the interplay between serotonin (5-hydroxytryptamine, 5-HT), circadian (24 hour) rhythms and depression and antidepressant drugs. The introduction, therefore, aims to provide an overview of the central serotoninergic system and factors affecting it, depression and the role of 5-HT in affective disorder and finally, the effect of antidepressant drugs on the 5-HT system and circadian rhythms.

Serotonin

Since its discovery in the central nervous system by Twarog and Page in the early 1950s serotonin (5-hydroxytryptamine, 5-HT) has been implicated in many forms of mental illness.

Serotonin can act as both an inhibitory and excitatory neurotransmitter and so 5-HT is thought to have a modulatory action in a variety of functions such as feeding, motor function, anxiety, sleep and hormonal release.

The central serotoninergic system arises from a group of cell bodies, which lie on or adjacent to the midline of the caudal brainstem through to the midline, called the raphe nuclei (RN). Formerly these nuclei were classified as B1-B9, but they are now divided into the superior and the inferior group (Jacobs and Azmitia 1992). The superior group, which comprises the dorsal and median raphe nuclei (DR and MR respectively), is the most important since it provides ascending innervation to virtually all brain regions. Whilst the inferior group projects to the ventral horn and substantia gelatinosa of the spinal cord.

Some target regions receive innervation from both the median and dorsal RN, but the degree of innervation can differ significantly, whilst others receive projections from only one of the RN, figure 1 shows a cross-section through the rat brain showing the location of the superior and inferior raphe groups and the preferential projections of the dorsal raphe (DR) and median raphe (MR) to regions of interest to this thesis.

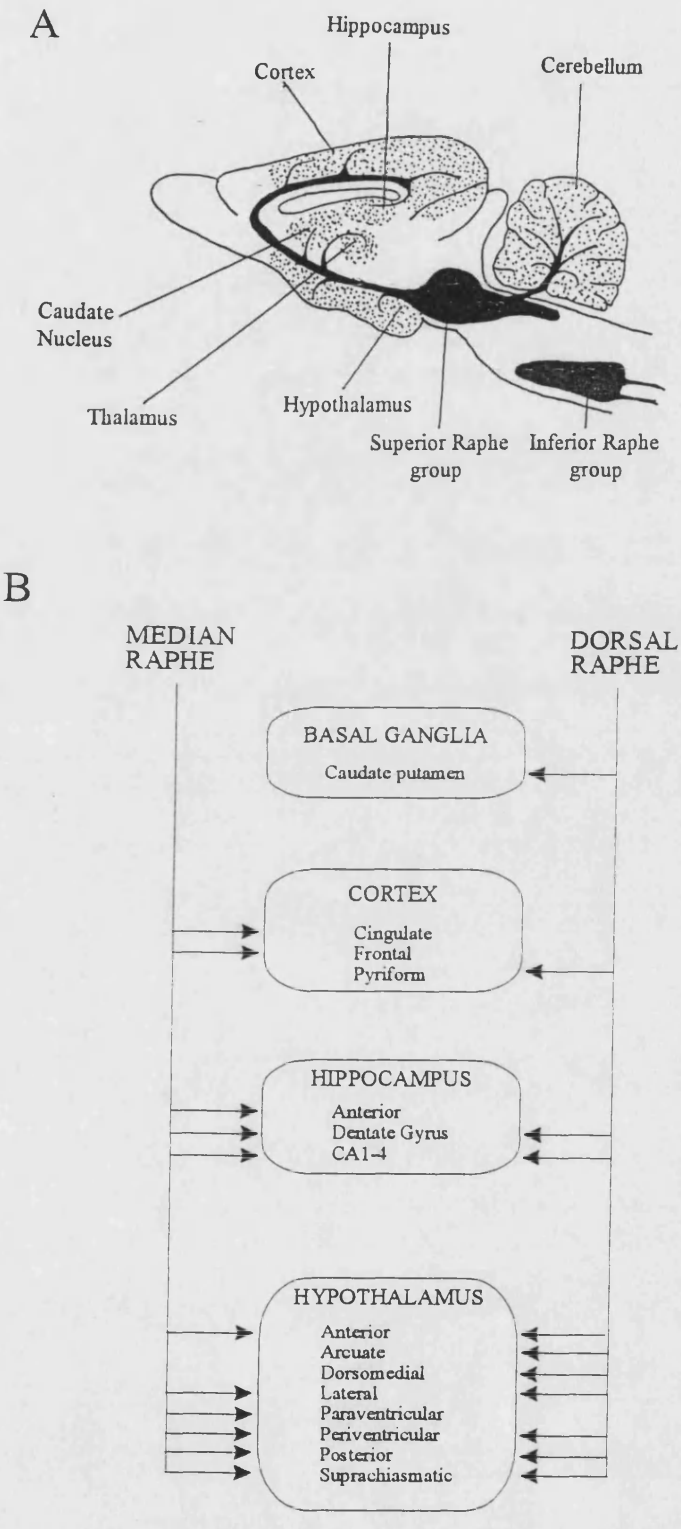


Figure 1. **A**. Coronal section through the rat brain indicating the location of the superior and inferior raphe nuclei. **B**. Schematic diagram of ascending 5-HT fibres from the median and dorsal raphe projecting to specific brain regions of interest to this thesis, based on Azmitia and Segal (1978).

There is evidence that the fibres that project from the DR and MR differ significantly (Tork 1990). Fibres from the DR are thin, branch diffusely in their target region, have small heterogenous varicosities ranging in size and make undefined synaptic contacts. In contrast, fibres originating from the MR are thick and non-varicose, but branches contain round or oval varicosities, and make well-defined synapses with target cells. The DR system is more sensitive to neurotoxins such as p-chloroamphetamine (PCA) and 3,4-methylenedioxymetamphetamine (MDMA) than the MR (Dewar et al. 1992).

The RN themselves receive a number of projections from other brain regions (neurotransmitters indicated in brackets), the superior vestibular nucleus (acetylcholine; ACh), the nucleus of the solitary tract (adrenaline), the locus coeruleus and subcoeruleus (noradrenaline; NA), the substantia nigra and ventral tegmental area (dopamine; DA) and the periaqueductal grey (neuropeptides). The MR and DR receive a major innervation from the lateral habenular nucleus which contains excitatory amino acids (EAA); this pathway synapses both directly onto MR and DR cell bodies and γ-aminobutyric acid (GABA) interneurons.

Serotonin synthesis

Since serotonin is hydrophilic at physiological pH, and therefore cannot cross the blood-brain barrier, it must be synthesised in the brain from dietary l-tryptophan (l-try); for a schematic diagram of the synthesis of 5-HT see figure 2. The level of circulating l-try is determined by the catabolic liver enzyme tryptophan pyrrolase and under normal conditions, 90% of plasma l-try is bound non-covalently to serum albumin; the d-isomer is not bound. L-tryptophan is actively transported across the blood-brain barrier by the neutral amino acid carrier, so the transport of l-try is open to competition from other neutral amino acids such as tyrosine and leucine. L-try is taken into neuronal cells and glia by a carrier-mediated and non-saturable diffusion process. Brain l-try levels vary between 1-10μM (Knowles and Pogson 1984). Newly accumulated l-try is considered to be preferentially converted to 5-HT, which has lead to the proposition that brain l-try is distributed to two compartments. The first is a high

capacity uptake system which accounts for 60% l-try influx into the brain, where the uptake is subject to competition from large neutral amino acids, the second a low capacity system which is non-competitive.

L-try is converted to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (EC 1.14.16.4, l-tryptophan; tetrahydropteridine oxygen oxidoreductase; TrOH), a mixed function oxidase which is only found in 5-HT synthesising neurons. The gene encoding brain TrOH has recently been cloned and sequenced (Kim et al. 1991). TrOH contains 444 amino acids, has a MW of about 51kDa and is 50% homologous to tyrosine hydroxylase, the rate limiting step in the biosynthesis of dopamine and noradrenaline. Greatest homology rests in the middle and C-terminal regions of the enzymes, indicating that these regions contain the catalytic domain whilst the N-terminal is responsible for substrate recognition. The conversion of l-try to 5-HTP is considered to be the rate-limiting step in the biosynthesis of 5-HT and requires molecular oxygen and reduced tetrahydrobiopterin (BH_4) as co-factors. Under normal conditions the enzyme is unsaturated since rat brain l-try levels range between 1 and 10μM and the K_m of TrOH for l-try is 50μM (Boadle-Biber 1993). In the conversion of l-try to 5-HTP one molecule of oxygen is reduced to water for hydroxylation at the 5 position, the electrons being donated by BH_4. The unstable quinoid dihydrobiopterin produced is immediately converted back to BH_4 by a NADH-linked quinoid dihydropteridine reductase.

Once 5-HTP has been synthesised it is decarboxylated to 5-HT by the non-specific aromatic amino acid decarboxylase (EC 4.1.1.28; AADC), almost immediately. The enzyme requires pyridoxal-5'-phosphate (vitamin B_6) as a co-factor. The 5-HT is formed in the cytoplasm and actively taken up by a specific 5-HT transporter driven by a pH gradient into dense core vesicles where it binds with high affinity to a specific serotonin-binding protein which is released with serotonin on exocytosis. Unsequestered 5-HT is degraded intraneuronally.

After 5-HT is released from the nerve terminal, and has acted on its target receptors, it is taken back into the neuron by a high affinity (K_m 0.1 to 0.5μM),

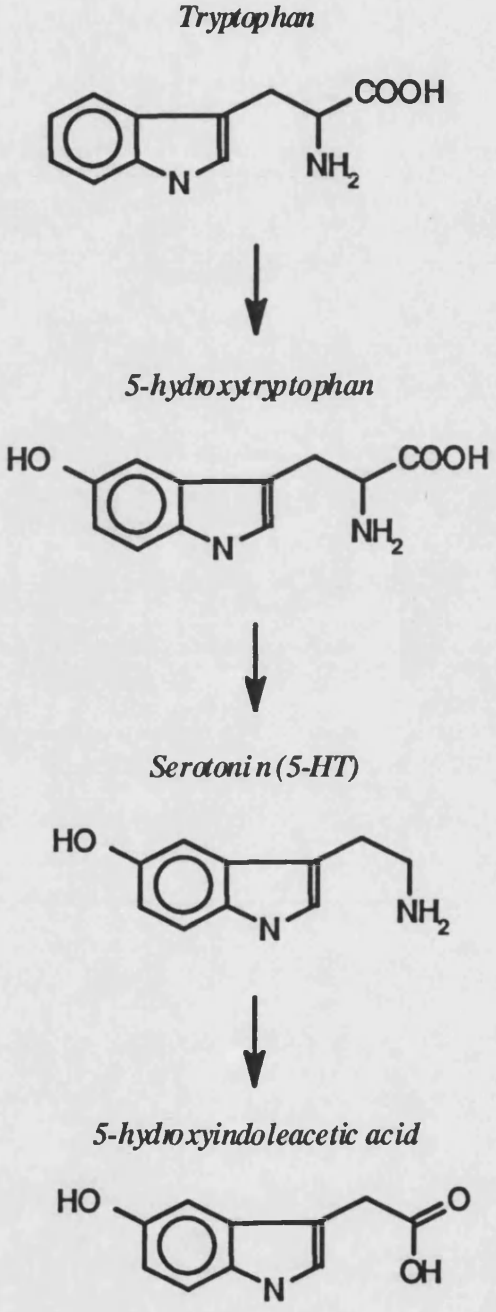


Figure 2. Schematic diagram of the pathway for the synthesis of 5-hydroxytryptamine from l-tryptophan in the brain. L-tryptophan, derived from the diet, is converted to 5-hydroxytryptophan by tryptophan hydroxylase. 5-Hydroxytryptophan is then decarboxylated by the non-specific l-aromatic amino acid decarboxylase to 5-hydroxytryptamine, which is, in turn, degraded to 5-hydroxyindoleacetic acid by monoamine oxidase.

specific sodium-dependent transporter (Shaskan and Snyder 1970), which has recently been cloned (Hoffman et al. 1991). Inside the neuron 5-HT is degraded to 5-hydroxyindole aldehyde (5-HIA) by monoamine oxidase (EC 1.4.3.4.; MAO), which is located on the external wall of mitochondria. There are two isoforms of MAO, A and B. Classically MAO-A and -B can be distinguished by their substrate preference and brain distribution. MAO-A oxidises serotonin, dopamine and noradrenaline and is found in catecholaminergic neurons, whilst MAO-B prefers trace amines such as phenylethylamine and methylhistamine and is located in serotoninergic neurons (Kwan et al. 1992; Richards et al. 1992). This apparent discrepancy has recently been addressed. It has been proposed that at physiological concentrations of 5-HT, MAO-A degrades trace substances that might interfere with the storage, release and degradation of 5-HT. Once 5-HIA is formed it is degraded to either 5-hydroxy-trytophol by alcohol dehydrogenase or 5-hydroxyindoleacetic acid (5-HIAA) by aldehyde dehydrogenase, depending on the energy state of the neuron. 5-HIAA moves across the blood brain barrier by an acid transport process and is excreted in the urine.

Factors affecting serotoninergic neurotransmission

The degree of control, and relative importance, that any of the factors listed below exert on 5-HT neurotransmission is currently under debate.

Precursor availability

One factor that may control the synthesis rate of 5-HT in the brain is the availability of its precursor l-try. *In vitro* studies using hypothalamic slices have shown that increasing the perfusing concentration of l-try increases both the basal and stimulated release of 5-HT (Schaechter and Wurtman 1990). This effect is dose-dependent (Lookingland et al. 1986) using l-try concentrations within the normal circadian range (Fernstrom and Wurtman 1971). These findings have been partially confirmed by *in vivo* studies. Peripheral injection of l-try can increase the level of 5-HT in dialysate samples in the ventral hippocampus of anaesthetised rats (Gartside et al. 1992a) or the cortex of freely moving rats (Carboni et al. 1986). Peripheral

administration of 5-HTP also increases dialysate 5-HT in the anaesthetised rat hypothalamus dose-dependently and the output was calcium-dependent (Gartside et al. 1992b). Interestingly decreasing the level of l-try, by increasing the concentration of other large neutral amino acids which compete with l-try for uptake, decreases 5-HT levels *in vitro* (Schaechter and Wurtman 1990) and *in vivo* (Gartside et al. 1992a). One study *in vivo* has shown no increase in dialysate 5-HT levels after l-try administration, although an increase in 5-HIAA was observed after the maximal dose of l-try (Sharp et al. 1992). However the amount of 5-HT released per pulse was increased after l-try administration and the increase was proportionally greater at higher stimulation frequencies of the DR (Sharp et al. 1992). Studies using positron emission topography in living dog brain have shown that increasing plasma l-try concentrations increases 5-HT synthesis rate in a linear fashion (Diksic et al. 1991), thereby providing convincing evidence that 5-HT synthesis rate can be affected by the plasma l-try level.

The function of the newly synthesised 5-HT is currently under debate. There is evidence that it is degraded intraneuronally (Lookingland et al. 1986; Sharp et al. 1992), spills out of the neuron (Trulson and Jacobs 1976), or is functional (Schaechter and Wurtman 1990; Gartside et al. 1992a; Sharp et al. 1992). It has been suggested (Sharp et al. 1992) that when the increase in l-try is within the circadian range the 5-HT produced is metabolised intraneuronally. At higher levels synthesis rate outstrips the degradation capability of the neuron and the 5-HT enters the releasable pool.

5-HT neuronal firing

Nearly all experiments investigating raphe 5-HT neuronal firing have been performed in unanaesthetised, head-restrained cats. Raphe neurons show a highly regular, slow discharge pattern in anaesthetised animals (Jacobs and Azmitia 1992) which appears to be an inherent property of the neurons since it persists *in vitro*. This rhythm is present from an early stage in development and can be accounted for by membrane ionic conductances (Jacobs and Azmitia 1992). The firing rate of the 5-HT neurons is related to the state of arousal of the animal, both in rats (Imeri et al. 1994) and cats (Trulson and Jacobs 1979; Wilkinson et al. 1991). The phenomenon has been

studied best in cats. During REM sleep cells fire at 0-0.3 spikes/sec; this increases in slow wave sleep to 0.3-1.5 spikes/sec , 1.5-2.0 spikes/sec when the cat is drowsy, 2.-3.0 spikes/sec during quiet waking, 3.0-5.0 spikes/sec during active waking and 4.0-7.0 spikes/sec when the cat is physically aroused. Using the technique of *in vivo* microdialsysis, Wilkinson and colleagues (1991) have demonstrated that changes in neuronal firing affect the terminal release of 5-HT. The firing rate of raphe 5-HT neurons is remarkably resistant to stimuli. Increased temperature or blood pressure, pain, changes in glucose availability, restraint or the introduction of a dog into the cat's vicinity all have no effect on the firing rate (Jacobs and Azmitia 1992). Stimulation of the RN has been reported to increase 5-HT synthesis (Petersen et al. 1989), whilst decreasing RN firing rate decreases synthesis (Invernizzi et al. 1991).

5-HT autoreceptors and heteroreceptors

An autoreceptor is a receptor located somatodendritically or terminally on a neuron and is only responsive to the neurotransmitter released from the same neuron. This is shown diagramatically in figure 3. If neurons A and B release the same neurotransmitter, the blue receptor located on neuron A is an autoreceptor for neuron A but a post-synaptic receptor for neuron B.

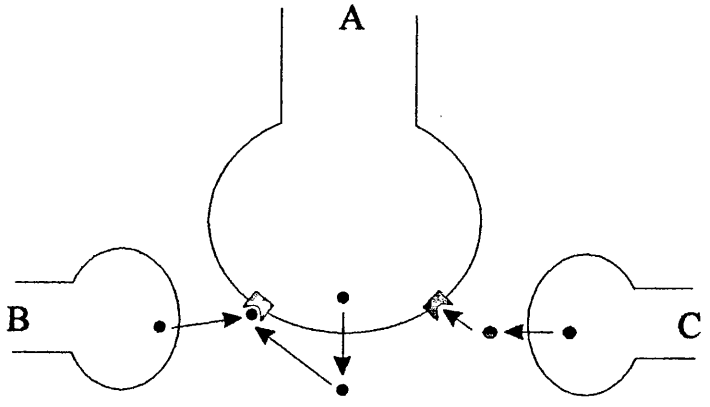


Figure 3. Diagram to show the distinction between autoreceptors and heteroreceptors. The blue receptor on neuron A is an autoreceptor for that neuron, but a post-synaptic receptor for neuron B. The green receptor on neuron A is sensitive to the neurotransmitter released from neuron C, but not sensitive to the neurotransmitter released from neuron A, and is therefore a heteroreceptor.

Cell body autoreceptor

The somatodendritic 5-HT autoreceptor in the RN has been characterised as a 5-HT_{1A} receptor (O'Connor and Kruk 1992). It is negatively coupled to adenylate cyclase and linked to the opening of a K^+ channel and closing of a Ca^{++} channel; stimulation of the autoreceptor therefore hyperpolarises the neuron. Electrophysiological recording of dorsal raphe cell firing *in vivo* (Fletcher et al. 1993) and *in vitro* (Lanfumey et al. 1993) has shown that the selective 5-HT_{1A} agonist, 8-hydroxy-2-(Di-*n*-propylamino)tetralin (8-OH-DPAT), decrease the neuronal firing rate in a dose-dependent manner. The effect can be antagonised by the novel, selective 5-HT_{1A} antagonist (±)WAY100135 (Fletcher et al. 1993; Lanfumey et al. 1993). Application of 8-OH-DPAT, either directly or peripherally, can decrease both the terminal release of 5-HT as measured by *in vivo* microdialysis (Hjorth and Sharp 1991; Bonvento et al. 1992; Routledge et al. 1993) or differential pulse voltammetry (Marsden and Martin 1986) and synthesis rate (Invernizzi et al. 1991; Fletcher et al. 1991) *in vivo*. The effect on release and synthesis rate are antagonised by WAY100135 (Fletcher et al. 1991; Routledge et al. 1993). Administered alone WAY100135 has no effects on 5-HT neuronal firing (Fletcher et al. 1993), indicating that 5-HT_{1A} autoreceptors do not display autoinhibitory tone. Thus the somatodendritic 5-HT_{1A} autoreceptor can influence 5-HT synthesis and terminal 5-HT release.

Terminal 5-HT autoreceptor

Farnebo and Hamberger (1974) were one of the first teams to demonstrate that 5-HT agents could decrease [^{3}H]5-HT released from cortical slices *in vitro*. Their findings were subsequently confirmed by other workers in hypothalamic synaptosomes (Cerrito and Raiteri 1979) and cortical slices (Gothert and Weinheimer 1979). All these workers showed that exogenously applied 5-HT decreased [^{3}H]5-HT release and that 5-HT_1 antagonists applied alone could increase this release and block the effects of 5-HT. Other 5-HT_2 antagonists tested e.g. cyproheptidine, methysergide, pizotifen and mianserin (Cerrito and Raiteri 1979; Baumann and Waldmeier 1981) were found to be ineffective, helping to define the receptor subtype mediating the inhibition.

Further characterisation of the terminal 5-HT autoreceptor as a 5-HT_1 receptor was performed by correlating ligand binding profiles with the ligand's effects on the stimulated overflow of [^{3}H]5-HT from hypothalamic synaptosomes (Martin and Sanders-Bush 1982). Since these workers used a synaptosomal preparation they could be sure that the effects of agonists were mediated by presynaptic receptors not an indirect effect due to stimulated release of another neurotransmitter from a different population of neurons which impinge upon, and affect, serotonin release. Subsequently the autoreceptor was defined as a 5-HT_{1B} receptor by the same techniques (Engel et al. 1986) and by superfusion alone (Middlemiss 1984; 1985).

The rat brain 5-HT_{1B} receptor has been cloned and characterised (Voigt et al. 1991). The receptor has a molecular weight of 43kDa, as has been reported for most G protein coupled receptors. The terminal 5-HT_{1B} autoreceptor is negatively coupled to adenylate cyclase (Bouhedal et al. 1988) by a pertussis-sensitive G-protein (Passarelli et al. 1988). Stimulation of the 5-HT_{1B} autoreceptor leads to a decrease in cyclic adenosine monophosphate (cAMP) and therefore reduced protein kinase A (PKA) activity. There are three protein kinase target sites within the third intracellular loop of the 5-HT_{1B} receptor, two for PKA at residues Thr 248 and Thr 309, and one for protein kinase C at residue Thr 243 (Voigt et al. 1991). The receptors are linked to adenylate cyclase by an inhibitory G-protein (G_i) which is composed of three subunits, α, β and γ. Under basal conditions the G protein is bound in a complex with guanine diphosphate (GDP). When 5-HT or other agonists bind to the receptor it causes a conformational change and the GDP dissociates. Guanine triphosphate (GTP) immediately binds to the site left vacant by GDP, which induces a conformational change and the complex of the α-subunit and GTP (α.GTP) dissociates from the neurotransmitter-receptor complex. The α.GTP is then free to inhibit adenylate cyclase. The α subunit possesses intrinsic GTPase action and catalyses the conversion of GTP to GDP. The resultant α.GDP complex has high affinity for the $\beta\gamma$ subunits and they reassociate to the basal state (Helper and Gilman 1992).

Cell body heteroreceptors

A heteroreceptor is a receptor that is not sensitive to the neurotransmitter released by the neuron on which it is present, see figure 3 above. Several heteroreceptors have been found to influence 5-HT neuronal firing; $GABA_A$ and $GABA_B$ (Becquet et al. 1990; Levine and Jacobs 1992), α_1-adrenoceptors (Baraban and Aghajanian 1980; Freedman and Aghajanian 1987) and $cholecystokinin_B$ receptors (Boden et al. 1991).

Terminal heteroreceptors

Several heteroreceptors located on serotoninergic terminals which can modulate 5-HT release have been identified e.g. α_2-adrenoceptors (Gothert et al. 1981; Tao and Hjorth 1992), further classified as an α_{2A}-adrenoceptor (Gobbi et al. 1990; Hjorth and Tao 1992), $GABA_B$ (Schlicker et al. 1984; Gray and Green 1987), H_3-receptors (Schlicker *et al.* 1988), muscarinic and nicotinic acetylcholine receptors (Hery et al. 1977a; Ennis and Cox 1982; Marchi et al. 1986), NPY (Schlicker et al. 1991). Stimulation of all these heteroreceptors decreases the output of 5-HT.

Circadian rhythms

In its true definition, the term "circadian" is used to define rhythms which oscillate over 24 hours in the absence of any external cues, zeitgebers. However, circadian is often used to describe rhythms which vary over 24 hours in the presence of a light-dark cycle, the major zeitgeber in experimental animals. Almost every aspect of 5-HT function, from synthesis to release, has been shown to vary over 24 hours. The circadian aspects will be divided according to their position in the scheme of synthesis and turnover. For simplicity I have only described peaks and troughs as being in the dark phase or in the light phase, for precise timings see table 1 at the end of this section.

L-Tryptophan

The level of free l-try in plasma have a circadian rhythm, although conflicting zeniths and nadirs have been published. The level is either maximal early in the light phase and minimal early in the dark phase (Redfern and Martin 1985) or peaks at mid

dark with the trough occurring at mid light (Hery et al. 1977b). The rate of l-try transport across the blood-brain barrier has not been measured over 24 hours. Since transport is a competitive process it might be assumed that the amount of l-try entering the brain is determined by its ratio to competing large neutral amino acids.

The uptake of l-try into cortical synaptosomes shows a distinct pattern, the peak K_m being at the transition from light to dark and the trough was 3 hours after lights off (Loizou and Redfern 1986). The V_{max} peaked at the end of the dark phase and fell to a trough in the first 3 hours of the dark phase (Loizou and Redfern 1986). As the authors point out, since the normal plasma concentration range of l-try is 1 to 10μM, the uptake system would appear to buffer any fluctuation in l-try levels and keep a constant rate of entry of l-try into the neuron. The uptake of [^{3}H]-try into hypothalamic or brainstem slices is greater during the light phase (Hery et al. 1972). The same results was obtained after intracisternal administration of [^{3}H]-try. These results are consistent with the finding that the circadian rhythm in brain l-try is not dependent on diet. If the food supply is restricted to a few hours in the light period then the rhythm in brain l-try is not significantly different from mice allowed free access to food (Morgan and Yndo 1973).

In a comprehensive survey of l-try levels in homogenates from seven different brain regions at mid light and mid dark, Pietraszek and colleagues (1992) consistently found lower levels in the light phase. This is consistent with the findings of Hery and colleagues (1977b). The concentration of l-try measured in hypothalamic homogenates of individually housed rats (Greco et al. 1988) showed a circadian variation directly opposite to that observed by Redfern and Martin (1985).

Tryptophan hydroxylase

One of the initial indications that the synthesis of 5-HT displayed a circadian rhythm was the demonstration that the conversion of [^{3}H]try to [^{3}H]5-HT was lower during the dark phase (Hery et al. 1972). However the authors point out that the variation is due to a rhythm in the uptake of [^{3}H]l-try. The activity of TrOH was found by Kan and colleagues (1977) to display a circadian rhythm. Differing rhythms were

demonstrated in TrOH activity in sonicates from individual raphe nuclei and the striatum. Their findings have been questioned since brain sonicates are thought to contain an endogenous inhibitor of TrOH (McLennan and Lees 1978). Since then some workers have found rhythms in TrOH activity in different brain regions; peak activity in the brainstem is during the light phase and lowest in the dark phase (Cahill and Ehret 1981), whilst pineal activity peaks in the night (Ehret et al. 1991). Others have failed to detect any rhythm in the striatum and midbrain (McLennan and Lees 1978). However, McLennan and Lees did find that the K_m of TrOH for BH_4 was 72% higher in the light phase. Measurement of the rate of accumulation of 5-HTP after its decarboxylation has been inhibited gives a good indication of the activity of TrOH. A significant variation in the rate of accumulation has been found in nine brain regions, however the position of the peaks and troughs differed significantly (Poncet et al. 1993). In the dorsal and central raphe nuclei, locus coeruleus, cortex and ventrolateral medulla both peaks and troughs occurred in the dark phase. In the raphe pallidus, paraventricular nucleus and suprachiasmatic nucleus (SCN) accumulation was greatest in the light phase. The dorsomedian medulla was unique in that peak accumulation occurred at the end of the dark phase (Poncet et al. 1993).

5-HTP decarboxylase

Only one study has assessed the activity of 5-HTP decarboxylase over 24 hours. A circadian rhythm in activity was demonstrated with a peak at 1700h and a trough at 0900h (Hillier and Redfern 1976). When pyridoxal phosphate, the co-factor, was added to the medium the enzyme's activity increased by 100%. The increase was the same at the two time points tested, implying that the rhythm observed was not due to differing levels of the active enzyme. The authors speculated that there could either be a variation in l-try entry into the neuron or that there is competition for the enzyme by other large neutral amino acids since the decarboxylase is not specific.

Tissue 5-HT level

Generally 5-HT levels have been found to be higher in the light phase *e.g.* in the hypothalamus, striatum and hippocampus (Pietraszek et al. 1992), SCN (Cagampang

and Inouye 1994), frontoparietal cortex (Hery et al. 1977b), central raphe nucleus and dorsomedian medulla (Poncet et al. 1993). 5-HT concentrations are highest in the dark phase in the dorsal raphe nucleus, ventrolateral medulla and SCN (Poncet et al. 1993), with the amplitude being greatest in the SCN.

5-HT release

The release of 5-HT is greatest in the dark phase when measured in the SCN (Faradji et al. 1983; Hutson et al. 1984; Martin and Marsden 1985; Glass et al. 1992), paraventricular nucleus (Martin and Marsden 1985) and hippocampus (Kalen et al. 1986) *in vivo*. This is in direct opposition to the peak synthetic activity suggesting that 5-HT is synthesised and then stored for release during the nocturnal animal's active period. However, *in vitro* [^{3}H]5-HT release was greater during the light phase (Blier et al. 1989). The difference may be accounted for by the experimental technique; Blier and co-workers compared the amount of [^{3}H]5-HT released after electrical stimulation at only two time points, one in the light phase and one in the dark phase. There is a circadian rhythm in the uptake of [^{3}H]5-HT (Meyer and Quay 1976) so more [^{3}H]5-HT may have been taken up at one time point than the other.

Reuptake of 5-HT

Meyer and Quay (1976) demonstrated a rhythm in [^{3}H]5-HT uptake into SCN slices and hypothalamic homogenates; peak uptake occurred at the transition from light to darkness and the trough at the transition from darkness to light. A circadian rhythm in the number of uptake sites has been demonstrated (Wirz-Justice et al. 1983). These workers found there were more uptake sites during the dark phase. Both these results suggest that uptake of 5-HT is greatest when release is highest. However the ligand used for binding studies, imipramine, is now considered to label a heterogenous population of uptake sites, one high affinity (the 5-HT uptake site) and one low affinity (Marcusson and Boss 1990).

5-HIAA concentration

5-HIAA levels in the cerebellum, hypothalamus, striatum, hippocampus and cortex are lower during the light phase (Pietraszek et al. 1992; Imeri et al. 1994). In the

frontoparietal cortex, though, levels are at their maximum early in the dark phase and minimal late in the light phase (Hery et al. 1977b).

Receptor rhythms

A circadian rhythm in the number of 5-HT_{1A} and 5-HT_{1B} binding sites has been demonstrated in the cerebral cortex (Akiyoshi et al. 1991; Weiner et al. 1992). In addition a seasonal rhythm in 5-HT_1, 5-HT_{1A} and 5-HT_2 binding has been shown (Weiner et al. 1992). The number of 5-HT_{1A}, 5-HT_{1B} receptors is higher during the dark phase than the light phase (Prosser et al. 1993), however this study did not distinguish between pre- and post-synaptic 5-HT_{1B} receptors. The strength of the *in situ* hybridisation signal for 5-HT_{1A}, 5-HT_{1B} ,5-HT_{2C} and 5-HT_2 receptors in the SCN has been found to be the same when measured at four time points in the light-dark cycle (Roca et al. 1993). Although this does not preclude a variation in membrane receptor numbers.

Serotoninergic neurons in the SCN, lateral geniculate nucleus (LGN) and hippocampus display a circadian rhythm in their sensitivity to iontophoretically applied 5-HT (Mason 1986), with a greater reduction in neuronal firing during the night. The recovery of the neuron from the 5-HT-ind

ed suppression of firing also differed significantly. Recovery was more rapid near the onset of the subjective night and slowest near the onset of the subjective day (Mason 1986). This is probably linked to circadian rhythm in the uptake of 5-HT (Meyer and Quay 1976). Depletion of 5-HT by parachlorophenylalanine (90-95%), which spares the actual 5-HT neurons, did not affect the circadian variation in sensitivity (Mason 1986). However electrolytic lesion of the SCN abolished the variation in sensitivity and decreased the sensitivity to iontophorised 5-HT in the LGN and hippocampus.

Table 1. Summary of published data on circadian rhythms in 5-HT function

Parameter	*Peak*	*Trough*	*Brain region*	*Reference*
tryptophan	free CT17 total CT21	free CT6 total CT10	plasma	Hery *et al.* 1977b
	free CT1 total CT1	free CT7 total CT13	plasma	Redfern and Martin 1985
Tissue tryptophan	CT6 CT6	CT18 CT12-	brainstem slice hypothalamus slice	Hery *et al* 1972
	CT20	CT10	cerebral cortex sonicate	Hery *et al.* 1977b
	CT12	CT1	hypothalamic homogenate	Greco *et al.* 1988
uptake l-try	 CT23.5 CT23.5	 CT15 CT15	cortical synaptosome V_{max} K_m	Loizou and Redfern 1986
TrOH activity	CT8 CT8 CT8	CT16 CT14 CT12	brainstem hypothalamus cortex	Hery *et al.* 1972
TrOH activity	CT1 CT8	CT11 CT18	dorsal raphe slice striatal slice	Kan *et al.* 1977
	lon +0h	loff +3h	brainstem homogenate	Cahill and Ehret 1981
	CT19 CT0 CT18 CT19 CT19 CT19	CT15 CT12 CT15 CT15 CT19 CT7	dorsal raphe ventral medulla dorsomedial medulla cortex paraventricular nucleus suprachiasmatic	Poncet *et al.* 1993

Parameter	*Peak*	*Trough*	*Brain Region*	*Reference*
Tissue 5-HT level	CT12	CT4/16	hypothalamic homogenate	Greco *et al.* 1988
	CT19	CT7	dorsal raphe	Poncet *et al.* 1993
	CT19	CT3/15	suprachiasmatic nucleus	
5-HT release	CT13	CT1	suprachiamstic nucleus	Faradji *et al.* 1983
	CT23	CT11	dorsal raphe	Agren *et al.* 1986
	CT23	CT11	locus coeruleus	
	CT2/10	CT8	hippocampus	Kalen *et al.* 1989
	CT14	CT11	paraventricular nucleus	Stanley *et al.* 1989
	CT14	CT8	suprachiasmatic nucleus	Glass *et al.* 1992
[^{3}H]5-HT uptake	CT11/15	CT12/18	hypothalamic homogenates	Meyer and Quay 1976
	CT12	CT3	suprachiasmatic nucleua slice	
Uptake sites	CT23	CT11	suprachiasmatic nucleus slice	Wirz-Justice et al. 1983
Binding			cerebral cortex	Akiyoshi *et al.* 1989
5-HT$_{1A}$	CT6	CT15		
5-HT$_{1B}$	CT3	CT18		

Table 1. Summary of the published data on circadian rhythms in the serotoninergic system. CT refers to circadian time after lights on in the animal colony if the animals were on a 12:12 light-dark cycle. In the case of Cahill and Ehret 1981, rats were subjected to an 8:16 light-dark cycle so the maximum and minimum is expressed as time after lights on (lon) or lights off (loff). Some authors referred to in the text have not been included in the table because the timing of lighting cycles was not included in their paper or they compared differences at only one time point in each phase of the light-dark cycle.

Depression

Depression is one of the most common forms of mental illness. The term depression covers a wide variety of illnesses and symptoms vary among patients. Sufferers complain of some or all of the following symptoms: hopelessness or helplessness; little interest in activities previously found pleasurable; fatigue; decreased concentration and thinking; indecision; worthlessness; excessive or inappropriate guilt; weight changes; sleep and circadian rhythm abnormalities.

There are two basic divisions of clinical depression; unipolar and bipolar. Unipolar depression affects, at any one time, 2-3% of males and 5-9% of females, and carries a lifetime risk of 8-12% for men and 20-26% of women (Horton 1991). Bipolar depression is less common, with a lifetime risk of 1% for both men and women. Unipolar sufferers show the symptoms described above, whilst bipolar depressives fluctuate between this state and bouts of mania. In the manic phase patients misperceive reality; hallucinate; hold bizarre beliefs and display strange behaviour; feel elated, carefree, overconfident and euphoric; overestimate their attractiveness, intelligence and abilities and possess unlimited energy. Bipolar depression shows a stronger hereditary trait, so offspring of bipolar depressives who themselves have depression are more likely to be bipolar depressives. Depression with physical symptoms and no apparent cause is termed endogenous depression, whilst reactive depression can be triggered by external events and anxiety.

Monoamine theory of depression

Thirty years after its formulation by Schildkraut the "monoamine theory of depression" is still commonly evoked to explain the aetiology of depression. The theory is based on an observation in the 1960s that patients treated with reserpine, a monoamine depletor, showed symptoms very similar to those observed in depression. The effects of reserpine could be blocked by pre-treatment with antidepressant drugs, which had just been discovered. However by no means all depressed patients respond to antidepressant drugs targeted to the central monoaminergic systems and it should be

borne in mind that the monoamine hypothesis is not the only theory concerning the cause of depression; other neurotransmitter candidates are ACh, DA and opiates. It is therefore possible that the symptoms of depression can arise as a result of a range of biochemical abnormalities.

Evidence for 5-HT involvement in depression

Having mentioned differing neurotransmitter hypotheses of depression, there is considerable evidence linking a malfunction in serotoninergic neurons with depressive illness. For instance there is a significant decrease in plasma tryptophan in depressed patients (Cowen et al. 1989; Pietraszek et al. 1991). Delgado and colleagues (1990) have shown that if the plasma level of total l-try in depressed patients in remission is rapidly depleted (by 87%) the majority of depressed patients relapse into depression. Their state improves gradually when they were returned to a regular diet, while control patients showed no response to l-try depletion (Delgado et al. 1990). The number of cortical 5-HT transporters is decreased in the cortex and hippocampus taken from depressed suicide victims (Leake et al. 1991; Little et al. 1993). The density of 5-HT_2 receptors in the frontal cortex and 5-HT_{1D} (the human equivalent of the rat 5-HT_{1B} receptor) receptors has been reported to be increased in depressed suicide victims (Stanley and Mann 1983; Lowther et al. 1991). However Cheetham and colleagues (1990) have shown that there is no change in hippocampal 5-HT_{1A} receptor binding in depressed suicide victims. Most binding studies in post-mortem human brain do not categorise the victim's type of depression. This, taken together with variations in post-mortem delay, age, sex, method of suicide may explain the differences in findings.

Another approach used is the neuroendocrine challenge test. This test depends on the ability of drugs which affect the 5-HT system to produce specific effects on plasma hormone levels. These effects cannot be specifically tied down to one step in the synthesis and turnover sequence of events. Administration of l-try decreases plasma prolactin and growth hormone (GH) levels. The l-try-induced decrease in plasma prolactin was even greater in non-melancholic depressed patients compared to healthy

volunteers, while in melancholic and psychotic depressed patients l-try administration increased plasma prolactin concentration (Price et al. 1991). The l-try-induced increase in plasma GH concentration was less in unipolar, non-melancholic and non-psychotic patients compared to control subjects (Price et al. 1991). The findings imply that changes in 5-HT function may differ according to the type of depression. Another neuroendocrine study has shown that the 5-HT_{1A}-mediated induction of adrenocorticotrophic hormone (ACTH) and cortisol secretion was less pronounced in unipolar depressives than controls (Lesch et al. 1990).

Circadian rhythms in depression

The term circadian describes a rhythm which oscillates over 24 hours, the rhythm is independent of external cues (such as a light-dark cycle) and will therefore persist in isolation.

Most, but by no means all, depressed patients complain of early morning wakening, which indicates a possible malfunction of the circadian system. In addition there is evidence of abnormalities in other circadian rhythms in some depressed patients, as outlined below. It is therefore possible that there may be at least one form of depression which is associated with circadian rhythm abnormality.

The amplitude of the circadian rhythm in plasma tryptophan is decreased in depressed patients compared to controls (Candito et al. 1992). In depressed patients, the acrophase (i.e. the peak level over 24 hours) in the noradrenaline metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) in plasma is phase advanced (de Met et al. 1984). Heart rate shows a significant circadian variation in normal subjects, however depressed patients show two distinct differences in this circadian variation. One group still possess a circadian rhythm but with a reduced amplitude, whilst a second group have no circadian rhythm (Taillard et al. 1990).

The circadian rhythm in the plasma concentration of prolactin, corticotrophin, cortisol, GH, thyrotrophin, ACTH, melatonin, as well as body temperature, heart rate sleep timing and structure have all been shown to be abnormal in depressed patients

compared to control subjects (Hallonquist et al. 1986; van Cauter and Turek 1986; Goetze and Tolle 1987; Daimon et al. 1992). The changes though have been reported variously to take the form of phase advance, phase delayed, and both reduction or increase in the amplitude of the rhythm. The differences may be due to the heterogeneity of the patients or difficulties inherent in collection of data. One of the most consistent finding is a phase advance in the onset of REM sleep, early morning wakening, peak ACTH and the nocturnal elevation in prolactin and GH (van Cauter and Turek 1986).

There are many hypotheses put forward to explain the circadian abnormalities which can be divided into two categories. The circadian system requires a pathway from the external environment to the pacemaker, the pacemaker itself and pathways from the pacemaker to target regions. The first set of hypotheses propose a variety of malfunctions of the pacemaker itself, e.g. internal desynchronisation, loss of amplitude, phase advance, inappropriate entrainment and dysregulation. In the second category Healy and Williams (1988) have hypothesised that there is no abnormality in the central clock, in fact a normally-functioning clock is a pre-requisite for the hypothesis, but that the transmission of information about the environment to the clock is abnormal.

Antidepressant Drugs

Antidepressants are clinically effective in alleviating depression only after repeated administration for more than about 14 days. There are 3 major classes of antidepressant drugs;

1. Uptake inhibitors: As the name implies these block the reuptake of neurotransmitters released into the synaptic cleft. The concentration of the neurotransmitter then builds up with consequent changes in pre- and post-synaptic receptor sensitivity and/or number. These can be non-specific e.g. amitriptyline which blocks the uptake of noradrenaline, dopamine, 5-HT and ACh or specific e.g. desipramine, which selectively blocks noradrenaline reuptake or nomifensine which is selective for dopamine reuptake. More recently highly selective inhibitors of 5-HT, termed SSRI (selective serotonin

reuptake inhibitors), have been introduced which are clinically very effective, e.g. paroxetine, fluoxetine, sertraline, fluvoxamine.

2. Monoamine oxidase inhibitors (MAOI): These prevent the breakdown of recaptured monoamines by irreversibly inhibiting the activity of MAO e.g. phenelzine, trancypromine. The administration of these drugs is hampered by quite serious side effects termed the "cheese reaction". Administration of MAOI blocks the activity of MAO in the gut wall and liver and therefore increases the circulating level of normally innocuous dietary amines, mainly tyramine. This results in a sympathomimetic effect, tyramine displacing noradrenaline from vesicles, leading to hypertension which produces headaches and even intracranial haemorrhage. Many foods contain tyramine, but one of the highest concentration is found in cheese. Within the last few years, selective monoamine oxidase-A inhibitors have been introduced. Called reversible inhibitors of monoamine oxidase-A (RIMA) they are claimed not to have the side effects of the old MAOI, an example is moclobemide.

3. "Atypical". These fall into neither of the above categories but are nevertheless clinically effective; their mechanisms of action are generally unknown (with the exception of mianserin, which is a 5-HT_2 antagonist), e.g. iprindole. The fact that "atypical" antidepressants are clinically effective appears to indicate that MAOI or blockade of reuptake is not essential for antidepressant action.

Effect of Antidepressant Drugs on the 5-HT system

Figure 4 shows a schematic diagram of 5-HT neurotransmission. Under normal conditions the effects of released 5-HT are terminated by re-uptake of 5-HT into the nerve terminal. Antidepressant treatment increases the synaptic level of 5-HT, termed the biophase concentration, by mechanisms depending on the class of antidepressant, as outlined above. Specific serotonin uptake inhibitors block the re-uptake of released 5-HT within a few hours of peripheral administration. MAOI block the degradation of

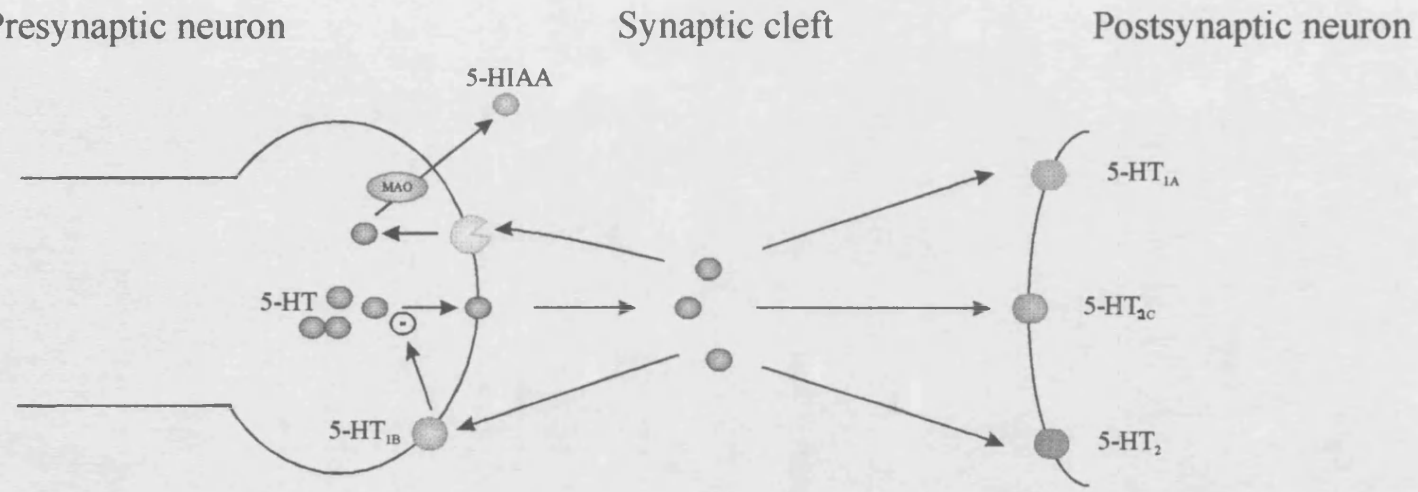


Figure 4. Schematic diagram of 5-HT neurotransmission. When 5-HT (blue sphere) is released from the nerve terminal it has several different sites of action. It can cross the synaptic cleft and bind to a variety of post-synaptic 5-HT receptors; 5-HT_{1A} (pink sphere), 5-HT_{2C} (orange sphere) or 5-HT_2 (green sphere). Alternatively it can bind to the presynaptically located 5-HT_{1B} autoreceptor (jade sphere). The interaction of 5-HT with its autoreceptor inhibits further 5-HT release. Transmission is terminated when 5-HT is taken back into the neuron by the 5-HT transporter (yellow pacman) and degraded by MAO (turquoise sphere) to 5-HIAA (purple sphere).

5-HT intraneuronally and 5-HT therefore spills out of the neuron. The acute effect of both types of antidepressant affects 5-HT levels in the terminal region by two actions. Firstly at the cell body level, the increase biophase concentration of 5-HT in the RN causes stimulation of cell body 5-HT_{1A} autoreceptors which decreases neuronal firing (Blier and de Montigny 1983; Chaput et al. 1986). In the terminal region the increased biophase 5-HT level after antidepressant administration stimulates 5-HT_{1B} autoreceptors, activation of which further inhibits 5-HT release. As a consequence of this increased biophase 5-HT level and concomitant repeated stimulation of the autoreceptors, it might be expected that the autoreceptors of both classes would desensitise like other classical neurotransmitter receptors. However this does not appear to be the case with 5-HT receptors (Maj and Moryl 1992; Newman et al. 1992). If the 5-HT_{1B} receptor is expressed in opossum kidney cell line, incubation with 5-HT produces maximal desensitisation of forskolin-stimulated adenylate cyclase and receptor down-regulation after 3 hours' incubation; both these parameters started to normalise after 48 hours' incubation (Pleus and Bylund 1992).

Whether autoreceptors down-regulate after chronic antidepressant treatment is pivotal in the action of antidepressants. Figures 5 and 6 show flow diagrams of the consequences of either down-regulation or no change in autoreceptor function. The experimental evidence provides support for both down-regulation and no change in autoreceptor function. The differences may be due to the diversity of antidepressants used, doses, dosing schedules, dosing methods, animal strains and method of assessing receptor function. After administration of 5-HT reuptake inhibitors 5-HT_{1A} and 5-HT_{1B} autoreceptors do desensitise (for 5-HT_{1A} see for example Blier and de Monitgny 1983; Chaput et al. 1986; Maj and Moryl 1992; Martin et al. 1993b; and for 5-HT_{1B} Blier and de Montigny 1983; Chaput et al. 1986; Moret and Briley 1990), although 5-HT_{1A} autoreceptor function has also been found to remain the same (Blier and de Montigny 1980). Indeed one behavioural test has even shown 5-HT_{1B} function to be enhanced after chronic 5-HT uptake inhibition (Maj and Moryl 1992). Again the increase in 5-HT concentration in the synaptic cleft would be expected to desensitise

post-synaptic 5-HT receptors; this has been demonstrated in some, but not all cases. 5-HT_{1A} receptor sensitivity has been reported to be unchanged (Blier et al. 1984; Chaput et al. 1986) or decreased (Blier et al. 1988) in the hippocampus using the same method but different antidepressants. The number of 5-HT_{1B} receptor binding sites, but not their affinity, is reduced in whole brain (Johanning et al. 1992), but receptor binding sites and affinity in the frontal cortex has also been found to be unchanged (Montero et al. 1991). Using behavioural measures, 5-HT_{2C} receptors have been shown to be desensitised (Maj and Moryl 1992). The number of 5-HT_2 binding sites in the frontal cortex is decreased (Peroutka and Snyder 1980) and behaviourally desensitised (Maj and Moryl 1992). Treatment with noradrenaline-selective or mixed function uptake inhibitors also affects 5-HT receptors, but again results are contradictory. Assessed by behavioural measures, 5-HT_{1A} autoreceptors are desensitised (Olpe et al. 1984; Lund et al. 1992) or supersensitive (Dijcks et al. 1991), whilst the number and affinity of post-synaptic 5-HT_{1A} receptors is decreased in the frontal cortex but unaffected in the hippocampus (Lund et al. 1992). The number, but not the affinity, is increased in the cortex (Martin et al. 1993) whereas the number, but not affinity, is decreased in the cortex but unchanged in the hippocampus (Pandey et al. 1991). The number or 5-HT_2 binding sites in the frontal cortex is decreased (Peroutka and Snyder 1980).

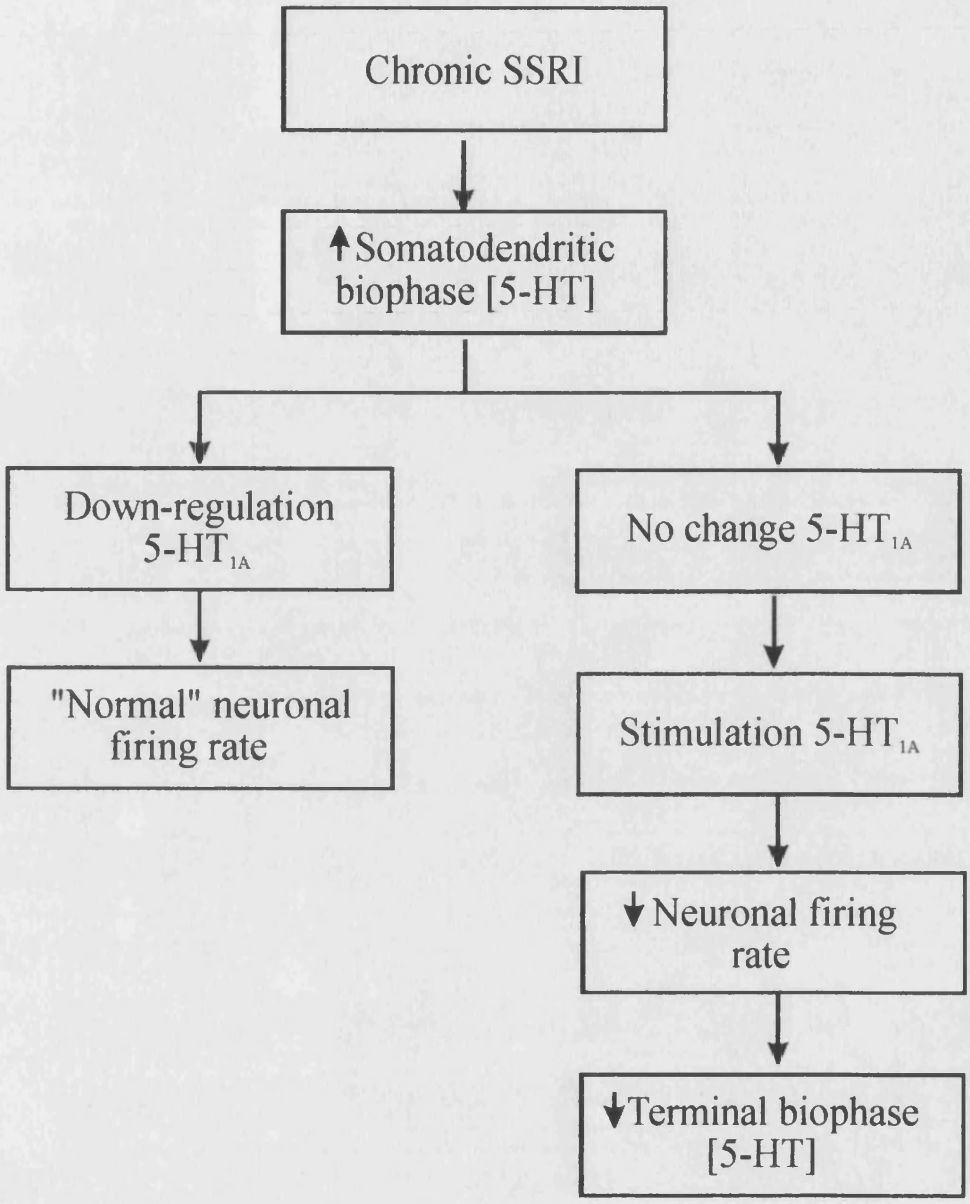


Figure 5. Flow diagram to show the effect of chronic antidepressant treatment on somatodendritic 5-HT_{1A} autoreceptors and the effect on terminal biophase concentration of 5-HT. Chronic specific serotonin reuptake inhibition (SSRI) increases the concentration of 5-HT in the raphe nucleus thus stimulation the 5-HT_{1A} autoreceptor. If the autoreceptor down-regulates, then 5-HT neuronal firing rate, and therefore release, remains the same. However if the autoreceptor's function remains the same then the firing rate of 5-HT neurons will decrease and the terminal release of 5-HT will also decrease.

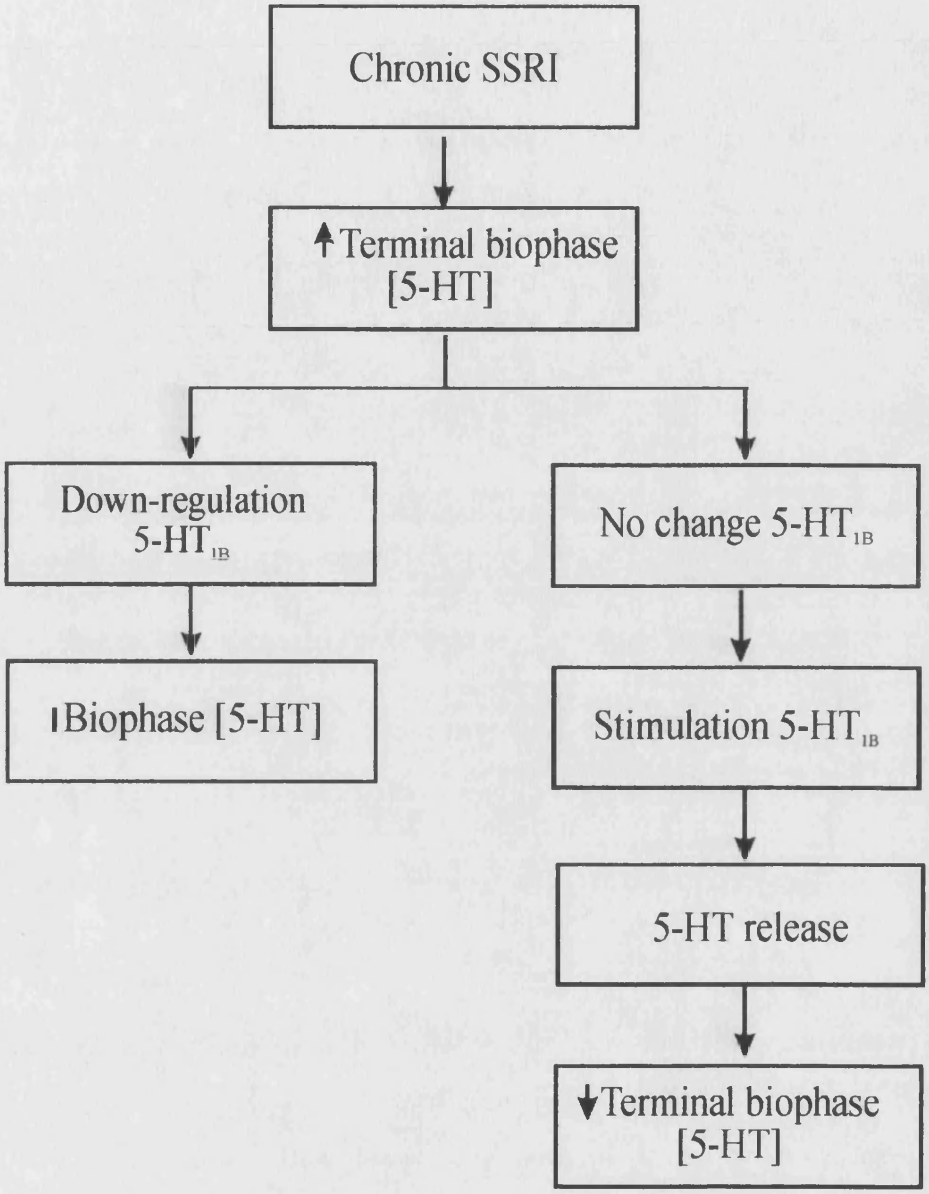


Figure 6. Flow diagram to show the effect of chronic antidepressant treatment on terminal 5-HT_{1B} autoreceptor function. Again, inhibition of 5-HT reuptake will increase the synaptic concentration of 5-HT in the terminal region. If the 5-HT_{1B} autoreceptor down-regulates, 5-HT release will be increased. However, if the function of the autoreceptor does not change then the biophase concentration of 5-HT will not be significantly different after uptake inhibition.

Effect of Antidepressant Drugs on Circadian Rhythms

There is evidence that prolonged administration of antidepressant drugs affects some circadian rhythms, although taken together the results are not clear cut.

The number of some neurotransmitter receptor populations varies over 24 hours *e.g.* α- and β-adrenoceptors, muscarinic acetylcholine receptors (mAChR), GABA-benzodiazepine receptors, α-melanocyte-stimulating hormone (αMSH) and dopamine receptors (Kafka et al. 1981a, b; O'Donahue et al. 1982; Wirz-Justice et al. 1982; Liebowitz et al. 1984; Jhanwar-Uniyal et al. 1986). Non-selective uptake inhibitors and MAOI phase delay the rhythm in α-adrenoceptors, GABA-benzodiazepine, αMSH, DA and mAChR receptors (Kafka et al. 1981b; O'Donahue et al. 1982; Wirz-Justice et al. 1982).

A 5-HT/noradrenaline uptake inhibitor, imipramine, has been reported both to accelerate the rate of re-entrainment of locomotor activity rhythms after a reversal of the photoperiod (Baltzer and Weiskrantz 1975) and to have no effect on the latency of re-entrainment to a 6 hour phase advance of the light-dark cycle in hamsters (Refinetti and Menaker 1993). The same antidepressant can significantly reduce the amplitude of the rhythm in plasma corticosterone in rats without affecting the acrophase i.e. timing of the peak (de Franciscis et al. 1987). However, in the same experiment, imipramine had no effect on the urinary levels of 5-HT and NA and their metabolites over 24 hours, though it must be noted that most of the 5-HT and 5-HIAA probably originated from the gut (de Franciscis et al. 1987). Clomipramine, a selective 5-HT uptake inhibitor and desipramine, a noradrenaline uptake inhibitor, did not affect the circadian rhythm in serum corticosterone and melatonin in the rat (Brown and Seggie 1988). Clomipramine hastened the re-entrainment of the temperature acrophase to a 6 hour phase advance of the light-dark cycle (Brown and Seggie 1988), whilst desipramine did not affect the rate of re-entrainment.

Desipramine has been shown to shorten the circadian period (tau, "day" length) of hamsters housed in constant darkness (Klemfuss and Kripke 1993) whilst a MAOI, clorgyline, increased tau (Wirz-Justice and Campbell 1982; Duncan et al. 1988).

Clorgyline treatment also delayed activity onset and offset in hamsters that were entrained (Duncan et al. 1988) or in constant darkness (Tamarkin et al. 1983). Moreover, chronic clorgyline treatment delayed hamster's re-entrainment to a four hour advance in the onset of its dark phase (Tamarkin et al. 1983). The amplitude of the free-running activity of rats kept in constant darkness has been shown to be increased by chronic treatment with desipramine, a noradrenaline uptake inhibitor and moclobemide, a RIMA. The free-running period was shortened by both these antidepressants (Wollnik 1992). These two parameters were unaffected by other antidepressants such as 5-HT uptake inhibitors and a MAO B inhibitor (Wollnik 1992). Interestingly Wollnik (1992) also demonstrated that desipramine and moclobemide change the intrinsic pattern of wheel running activity of Lewis rats. Before treatment, the nocturnal activity was divided into distinct short activity bouts about 4-5 hours apart. Prolonged antidepressant treatment unified the multimodal activity pattern. The mechansim and/or site of action for this effect is unknown.

Summary

The central 5-HT system is diffuse, projecting to virtually every brain region. The release of 5-HT is controlled by many factors including neuronal firing rate, somatodendritic and terminal autoreceptors and a circadian rhythm in all aspects of 5-HT function. There is much evidence to suggest an abnormalality in 5-HT function as the aetiology of depression. Additionally, there is evidence for a circadian rhythm malfunction in some forms of depression. Many clinically effective antidepressant drugs target the 5-HT system and can affect circadian rhythms. Taken together the results of these studies could indicate that antidepressant drugs may be alleviating depression by affecting circadian rhythms and that this effect is mediated through the 5-HT system.

Project Aims

The hypothesis is proposed that the efficacy of antidepressant drugs stems, in part, from their ability to differentially alter the degree of control the terminal 5-HT_{1B} autoreceptor exerts on synthesis and release through the light-dark cycle. This hypothesis is based on the assumption of a link between a deficiency of serotoninergic activity and circadian rhythm malfunction in depression, the fact that many clinically effective antidepressant drugs affect the central 5-HT system and can be shown to affect circadian rhythms and given that central serotoninergic function is itself subject to circadian variation. The function of the terminal autoreceptor is of paramount importance because it influences the biophase concentration of 5-HT and therefore the amount of 5-HT reaching its post-synaptic targets.

The hypothesis was tested by studying the effect 5-HT_{1B} autoreceptor stimulation exerted on release, using *in vitro* superfusion and *in vivo* microdialysis, and on the rate of 5-HT synthesis, by measuring 5-hydroxytryptophan accumulation. The effect of receptor stimulation on these two parameters was examined at four, equally spaced times through the light-dark cycle and after chronic antidepressant treatment.

A review of the techniques used in this thesis is given in the next section.

Techniques Appraisal

Superfusion

In this study the continuous superfusion technique developed by Frankhuyzen and Mulder (1982) to measure [^{3}H]noradrenaline ([^{3}H]NA) release from cortical slices *in vitro* as adapted by Middlemiss (1984) was used.

The technique of Frankhuyzen and Mulder was designed to produce rapid and accurate cumulative dose response curves for drugs affecting [^{3}H]NA release in the CNS; the principle being essentially the same as that used for isolated peripheral organ preparations. Frankhuyzen and Mulder compared [^{3}H]NA release during multiple 20mM K^+ pulses or continuous perfusion of 20mM K^+-containing Krebs buffer. They found that the amount of [^{3}H]NA released after repetitive stimulation was similar in size to that found in the corresponding collection periods during continuous stimulation. In both cases the release consisted of at least 85% unmetabolised [^{3}H]NA. The results using, different stimulation methods, showed no differences in the calcium-dependence of the release, or inhibition of [^{3}H]NA release by autoreceptor stimulation. Both techniques showed that NA produced maximal inhibition of [^{3}H]NA release after 10 minutes and that the magnitude of the inhibitory effect of NA was independent of the time of its addition to the superfusing buffer, indicating that the sensitivity of the autoreceptor remained unchanged. Moreover the recovery from NA-induced inhibition, i.e. when NA was removed from the buffer, was the same.

Middlemiss (1984) slightly adapted the technique i.e. by inclusion of pargyline (to prevent degradation of the radiolabel by MAO) and paroxetine (to block re-uptake of 5-HT and thus displacement of the radiolabel) in the superfusing buffer, and used it to measure [^{3}H]5-HT release from cortical "chips". He found that the [^{3}H]5-HT efflux rate in the absence of exogenous 5-HT had declined by 32% compared with the value at the beginning of the experiment. Using a range of 5-HT concentrations (30, 100, 300 and 1000nM) Middlemiss determined that maximal 5-HT inhibition of [^{3}H]5-HT release occurred at 1000nM, which decreased [^{3}H]5-HT output by 68%. The apparent IC_{34} of the autoreceptor for exogenous 5-HT (i.e. the concentration of 5-HT added

that decreased [^{3}H]5-HT release by half, referred to from here simply as the IC_{50} for comparison with published data), calculated using the cumulative dose-response technique was 46nM.

The technique described above was used in this thesis to measure [^{3}H]5-HT release from hypothalamic "chips". The technique offers the major advantage of reducing the number of animals used to construct dose-response curves and to calculate pA_2 values. As with all *in vitro* studies, the isolation of the tissue from neuronal and humoral factors that might affect the neuron's activity could affect the response of the neuron.

In vivo microdialysis

The first dialysis model was produced by Bito and colleagues in the mid 1960s. It consisted of a permeable sack 8-12mm long and filled with 6% dextran in saline. Unfortunately, however, only one sample could be obtained per probe. The technique of repeated sampling was developed by Delgado and co-workers in the early 1970s. In 1972 they published a method for assessing neurotransmitter levels in living, conscious Rhesus monkeys for up to 8 months. It was an adaptation of the push-pull cannula technique, which they termed a transdermal dialytrode since it could incorporate a recording/stimulating electrode. The transdermal dialytrode, see figure 7, was totally subcutaneous. The probe was formed from two pieces of teflon tubing cemented together, with one tip ending 1mm shorter than the other; both tips were enclosed in a polysulphone bag (3µl). The other ends of the tubing terminated separately in two rubber reservoirs. The system was continuously superfused and drugs could be injected into one bag and samples collected from the other.

U-shaped and transcerebral probes were developed by Zetterstrom and Ungerstedt in the early 1980s. These workers were also one of the first groups to couple the technique to the highly sensitive detection ability of high performance liquid chromatography (HPLC) linked to either electrochemical or fluorometric detection. It was only with the advent of HPLC that the full potential of measuring

neurotransmitters from small samples of extracellular fluid could be exploited. Transcerebral and U-shaped probes are not suitable for use in deep brain structures such as the hypothalamus, since their implantation causes too much tissue damage. The three basic designs of dialysis probe currently used are shown in figure 7.

Microdialysis offers several advantages over other neurochemical techniques. The dialysis probe can reflect rapid changes in the extracellular neurotransmitter levels (Auerbach et al. 1989). Probe implantation produces minimal tissue damage, it is a closed system producing clean samples which do not require lengthy preparation before analysis and it can be used in anaesthetised and freely moving animals to measure extracellular neurotransmitter levels not their metabolites. It is debated whether measuring the levels of the 5-HT metabolite 5-HIAA actually represents 5-HT release (see for example Crespi et al. 1990). The probe is stereotaxically implanted into the specified brain region with reference to one of two specific points, Bregma and lambda, on the surface of the rat's skull as shown in figure 7.

It is important to realise that the neurotransmitter measured in the dialysate does not directly represent synaptic release; rather it measures the amount of neurotransmitter that diffuses into the area around the tip of the probe. To establish that the neurotransmitter measured is neuronally-derived three criteria should be fulfilled. The output should be calcium- and tetrodotoxin-dependent and should be increased after high K^+ (30-100mM) infusion (di Chiara 1990).

The recovery of the probe, i.e. the amount of neurotransmitter that diffuses into the probe, is dependent on several factors. Increasing the perfusion rate significantly alters the recovery rate. Rates of 0.2-2µl/min produce small but highly concentrated samples and the recovery can be as high as 85% (Benviste 1989); faster perfusion rates (2-10µl/min) produce larger, more dilute samples with a recovery of only 2-10%. Higher flow rates may produce positive hydrostatic pressure gradients across the membrane which decreases mass transport across the membrane. Filtration forces are also minimal if the flow rate is kept low. Temperature significantly affects recovery rates; there is a 1-2% increase in recovery for every 1 °C increase in temperature.

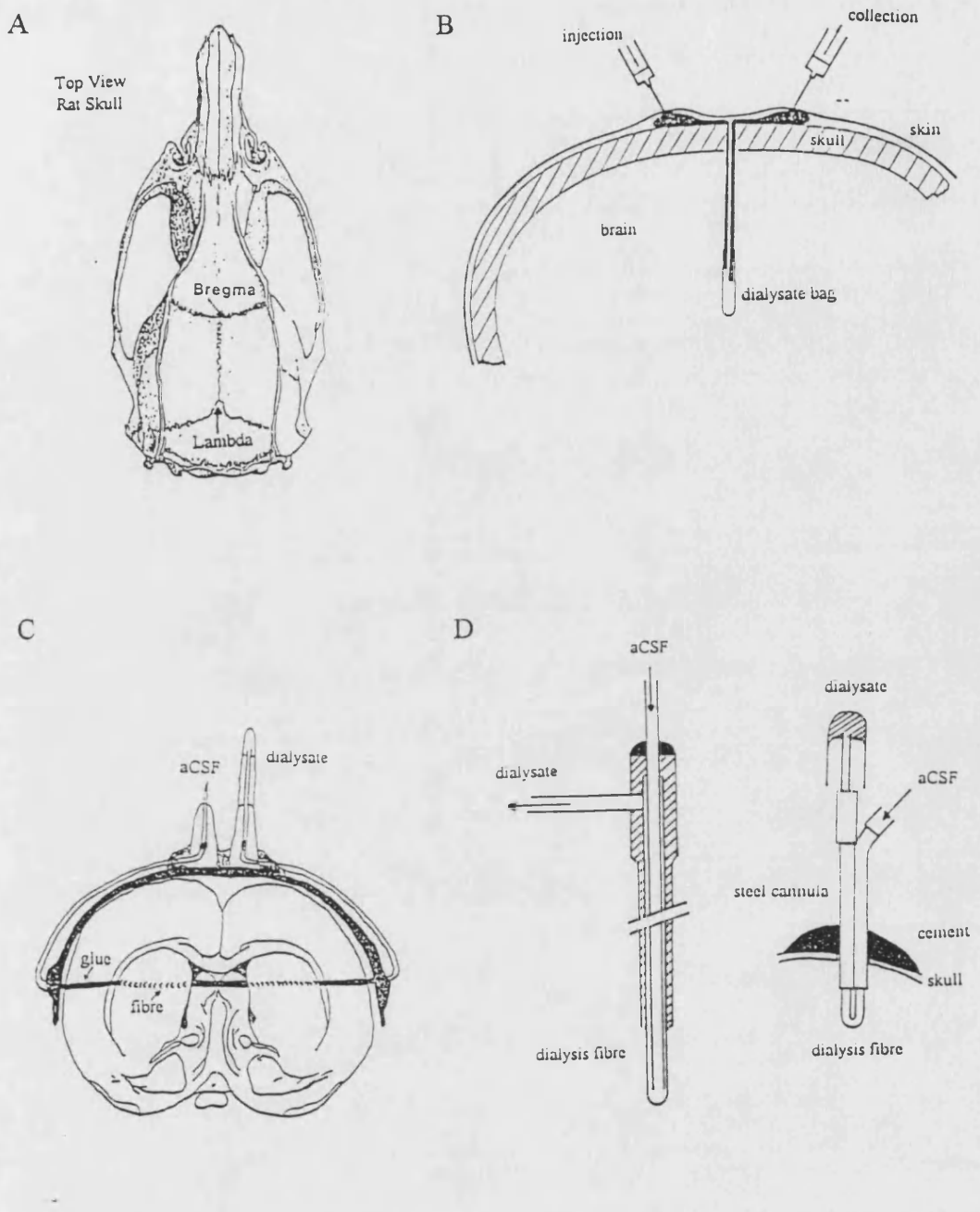


Figure 7. Cross-section through different types of microdialysis probe. **A**. Top view of a rat's skull showing the position of the two reference points, Bregma and lambda, used for stereotaxic implantation of microdialysis probes, adapted from Paxinos and Watson (1982) . **B**. Picture of Delgado and colleague's original transdermal dialytrode taken from their 1972 paper; for description see text. **C**. Transcerebral and **D**. left concentric and right U-shaped microdialysis probes, redrawn from di Chiara 1990.

Recovery is also dependent on the membrane area, obviously the larger the area for diffusion the greater the recovery. It is therefore important to measure the recovery of the probe before each use, see microdialysis methods section on page 120.

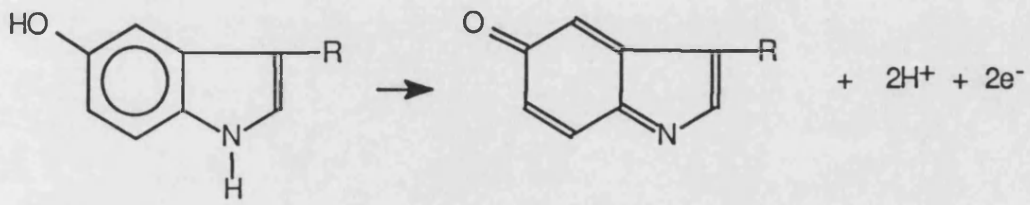
There are two potential problems with the technique *in vivo*. The pathway to the dialysis probe can be impeded by the cell membrane which would affect the probe recovery. Secondly continuous sampling of the neurotransmitter, especially after uptake inhibition, may limit the supply of the neurotransmitter with time. It is also essential that the blood brain barrier remains intact after probe implantation to avoid contamination from the systemic circulation; this is obviously very important when measuring 5-HT. Experiments should therefore not be performed less than 10 minutes after implantation because the blood brain barrier is not intact and tissues are reacting to the trauma of probe implantation.

Not very much is known about application of drugs via the probe. Theoretically it would depend on the drug concentration, drug interaction with the membrane, temperature, diffusion coefficient of the drug and its uptake into intracellular and vascular compartments. If drugs are administered peripherally they may not penetrate the blood brain barrier and exact site of action in the brain cannot be defined. The changes in neurotransmitter reflected in the dialysate therefore may be due to direct effects in that area or by affecting other neurotransmitter systems which project to the area under investigation. In addition the concentration of drug at the receptor site can never be estimated. By administering drugs via the probe the effect of the drug can be localised and the concentration of drug reaching the receptor site estimated; additionally peripheral effects of the drugs can be avoided.

Microdialysis takes advantage of the sensitivity of HPLC, in this study reverse phase HPLC linked to electrochemical detection was used. The principles of electrochemical detection are outlined below.

Reverse-phase refers to the fact that the stationary phase is non-polar and the mobile phase is polar. The stationary phase is composed of fine silica particles with bonded hydrocarbon alkyl chains, in this case octadecyl. An ion-pairing agent was

included in the buffer to increase the hydrophobicity of molecules and therefore decrease their rate of passage along the HPLC column. The constituents of the supernatants are separated along the column according to their molecular size and structure. As the eluant from the column is passed over a glassy carbon electrode set at an optimum potential for 5-HT oxidation, each molecule of 5-HT is oxidised to 5-ketotryptamine and two electrons are released, as shown below. The current generated by the released electrons is detected by a reference electrode that deflects the pen of the chart recorder to give a characteristically-timed peak. The greater the concentration of 5-HT, the more electrons are released and thus the greater the pen's deflection on the chart recorder.



5-Hydroxytryptophan (5-HTP) Accumulation

Inhibitors of aromatic-L-amino acid decarboxylase (AAAD) are a useful tool in neurochemical research. By inhibiting the conversion of 5-HTP to 5-HT, see page 4, these chemicals allow the accumulation of 5-HTP. The extent of this accumulation can be used to measure the rate of tryptophan hydroxylation with time. It has previously been reported that some AAADs inhibitors also inhibit TrOH (Johansen et al. 1991). However these workers found that m-hydroxybenzyhydrazine (NSD1015), the AAAD inhibitor used in this study, did not affect TrOH activity *in vitro* up to a concentration of 1mM.

Carlsson and colleagues (1972) developed the technique of AAAD inhibition as a direct measure of synthesis rate as an alternative to isotopic methods. These workers examined several aspects of NSD1015 inhibition of AAAD. They established that the accumulation of 5-HTP after NSD1015, at a dose of 100mg/kg i.p., was linear with

time up to 30 minutes after administration in the cerebral hemispheres and striatum. From 30 to 60 minutes the rate of accumulation seemed to decline slightly. Varying the dose of NSD1015 between 50, 100 and 200 mg/kg i.p. had no significant effect on 5-HTP accumulation, indicating that the enzyme was fully inhibited at a dose of 100mg/kg i.p.. If NSD1015 (100mg/kg i.p.) was administered and the levels of try and 5-HT measured for 40 minutes after administration then try levels remained level throughout that period but 5-HT were observed to drop slightly by 40 minutes after AAAD inhibition. The same results have been obtained from studies performed in our laboratory and therefore a dose of 100mg/kg i.p. NSD1015 and a post administration interval of 30 minutes were chosen as standard parameters. Bedard and colleagues (1971) have shown that the 5-HTP measured is in serotoninergic neurons.

The 5-HTP accumulation technique has the advantage of being able to measure the activity of the rate limiting enzyme in serotonin biosynthesis. However it is not so successful if used to measure tyrosine hydroxylase activity. The AAAD is a non-specific enzyme located in noradrenergic and dopaminergic neurons (as well as serotoninergic neurons). Therefore it cannot be determined if accumulated dihydroxyphenylalanine (DOPA), the common precursor of noradrenaline and dopamine, is derived from noradrenergic or dopaminergic neurons. Bearing this in mind, there is one other major drawback to the technique. Accumulated DOPA undergoes substantial efflux from neurons (Nissbrandt et al. 1988) and could enter 5-HT neurons and inhibit TrOH; although this inhibition has been shown to be weak after total AAAD inhibition (Johansen et al. 1991). Nissbrandt and colleagues (1988) have shown that DOPA levels can rise as high as 16μM after AAAD inhibition by NSD1015, but Johansen and co-workers (1991) demonstrated that TrOH activity *in vitro* is only significantly inhibited by DOPA concentrations in excess of 30μM. However NSD1015 is commonly used in 5-HT research since it has lesser effects on DOPA accumulation that other AAAD inhibitors (Nissbrandt et al. 1988).

Chapter 2 Superfusion of Hypothalamic Tissue

Hypothesis

In vitro superfusion studies were employed to investigate the hypotheses that in rats entrained to a light-dark cycle;

a) The function of the terminal 5-HT_{1B} autoreceptor varies over 24 hours.

b) Chronic antidepressant treatment would alter the function of 5-HT_{1B} receptors controlling [^{3}H]5-HT release.

c) The effect of chronic antidepressant treatment on autoreceptor function would be phase-dependent, i.e. differently over the light-dark cycle.

Method

Housing of Animals

Specially designed sound attenuated and light proof boxes were used to phase shift animals for circadian rhythm studies as previously described (Hillier et al. 1973). The walls of the boxes were insulated with polystyrene (2.4 cm thick) and draught excluder strips (1.3cm thick) were stuck to the edge of the cabinet door to exclude light. One extractor fan (Phillips, type HR 3408) provided ventilation for 4 boxes through a series of pipes. Each box contained its own, independently controllable fluorescent strip light (10cm, 100 lux at the top of the cage) with the choke removed and refitted outside the box to keep the boxes cooler. Lighting schedules inside each box were manipulated externally using timer switches (Smiths, TS 100 C).

Six male Wistar rats (bred at the University of Bath), initially weighing 150g, were placed in a standard animal cage inside each box for 14 days prior to use, to entrain their rhythms to their new light-dark cycle. This time is considered to be adequate for the re-entrainment of circadian rhythms to new lighting cycles (Hillegaart and Ahlenius 1994). In fact, locomotor activity was entrained to a new light-dark cycle within 5 days. Inside the box the rats had free access to food and tap water and could be left undisturbed between cleanings. They were cleaned out every 2-3 days and the

time of cleaning was randomised to avoid disturbance acting as a zeitgeber. The overhead lights in the animal room were never turned on when the animals were cleaned out. If cleaning was carried out whilst rats were in the dark part of their lighting cycle the room was illuminated by low intensity red light (4-6 lux) since this wavelength of light has been shown to have least effect on rat circadian rhythms (McGuire et al. 1973). The rats were handled individually for a few minutes to familiarise them to human contact at the same time as cage cleaning. The temperature inside the boxes ranged from 20-25°C.

Superfusion apparatus

Jacketed glass buffer reservoirs were connected to the well of the superfusion block by three-way taps to make the interchange between buffer solutions easier. Buffer was delivered to the ten wells of the superfusion block by a Watson-Marlow pump (model 502S). The superfusion block and buffer reservoirs were kept at 37°C by a water circulator (Grant) and the buffer was continuously gassed with 95%:5% O_2:CO_2. The tissues were superfused against gravity and effluent collected into scintillation vials using a fraction collector (Gilson, model 203, Anachem).

Tissue preparation

Male Wistar rats (University of Bath) weighing 240-260g were killed by cervical dislocation and the basal hypothalamus, including the SCN, rapidly dissected onto a cooled cutting disc. Bidirectional 250µm thick slices were cut on a McIlwain tissue chopper (Gomshall). The slices were incubated for 30 mins at 37°C in 5ml Krebs buffer (composition in mM; NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4.7H_2O$ 1.19, KH_2PO_4 1.18, $CaCl_2.6H_2O$ 1.30, glucose 2mg/ml) containing 0.1µM [^{3}H]5-HT (specific activity 10-20 Ci/mM, Amersham), 10µM pargyline, to prevent the breakdown of [^{3}H]5-HT, and 0.2mM ascorbic acid as an anti-oxidant. The slices were washed three times with 5ml Krebs buffer containing, in addition to the above, 3.2µM paroxetine, the specific 5-HT uptake inhibitor. Aliquots of tissue (usually 50µl) were

transferred to the wells of the superfusion apparatus and superfused against gravity with Krebs buffer. The tissue was left for 30 mins to equilibrate before the start of any experiment. At the end of the experiment the tissue from each well was collected and sonicated in scintillation fluid for 15 mins to dissolve the tissue and release the [^{3}H]5-HT taken up.

Uptake Studies

Two sets of uptake experiment were performed. The first was to determine the degree of non-specific uptake of [^{3}H]5-HT in the hypothalamus and the second to investigate the degree of uptake at the four time points chosen.

For the first experiment, two rats were killed and their hypothalami removed as described above. Both sets of brain tissue were incubated in 5ml Krebs buffer as above but without the [^{3}H]5-HT, one incubation medium also included 3.2µM paroxetine for 15 mins. After 15 mins 0.1µM [^{3}H]5-HT and ascorbic acid were added to both sets of tissue and the tissue incubated for a further 15 mins.

For the second experiment the hypothalamic tissue from animals killed in the appropriate phase of their light-dark cycle, in either normal lighting conditions or dim red light (4-6 lux) as necessary, was incubated in 5ml 0.1µM [^{3}H]5-HT for 30 mins.

In both experiments aliquots (100µl) of tissue, were transferred to each well of the superfusion system; each well contained a pre-weighed nylon gauze at the bottom. The tissue was superfused with Krebs buffer for 30 mins, the tissue and gauze from each chamber was removed and weighed, then added to a scintillation vial containing 10ml scintillation fluid. The vials were sonicated for 15 mins to solubilise the tissue, and left to stabilise for 3 hours prior to counting.

24 Hour rhythm studies

Rats which had been phase-shifted as described above were used for these experiments. In general rats were taken from each lighting schedule randomly to ensure that on one day no two rats taken from the same lighting schedule were used. There

was one exception however; two rats from the same lighting schedule were used on the same day if it would have entailed leaving one rat alone in a cage overnight.

Rats were killed at the appropriate phase of their light-dark cycle. The tissues were prepared as described above and left to stabilise in the superfusion chambers for 30 mins. In the 10 chambered superfusion block, two chambers served as controls (5mM K^+Krebs) throughout the experiment, whilst the other eight chambers received 25mM K^+ Krebs (achieved by isomolar substitution of NaCl for KCl). After two control fractions had been collected, four out of the eight chambers receiving 25mM K^+ were exposed to increasing concentrations of 5-HT (30, 100, 300 and 1000nM) delivered in 25mM K^+ Krebs buffer, therefore each manipulation was carried out in quadruplicate. Each concentration of 5-HT was exposed to the tissue for four fractions (16 min). Using this method 5-HT has previously been shown to have its maximal effect after 12 mins (Middlemiss 1984; Singh and Redfern 1994a).

Calcium Studies

Rat hypothalamic slices were incubated in 5ml of Krebs buffer containing 0.1μM [^{3}H]5-HT and 0.2mM ascorbic acid and treated as described above. Of the ten chambers, two were used as controls receiving normal (5mM K^+) Krebs throughout the experiment, the other chambers were superfused with Krebs buffer containing 25mM K^+ and two fractions were collected. For the rest of the experiment four chambers out of the eight received 25mM K^+ Krebs and four chambers 25mM K^+ Krebs buffer without Ca^{++} (isomolar replacement of $CaCl_2$ with $MgCl_2$). Ten consecutive fractions were collected and their radioactive content determined by liquid scintillation counting.

Drugs

5-HT creatinine sulphate (30-1000nM) was made up in the normal superfusion buffer containing pargyline and paroxetine, gassed with 95:5 O_2:CO_2 and kept at 37° C. 5-HT was administered by switching the three way tap to the buffer reservoir

containing 5-HT, having drained part of the 5-HT-containing buffer which had become cold in the tube and being careful not to introduce air bubbles into the tube since they would disrupt the release of [^{3}H]5-HT. The timing of the buffer switch over was calculated to allow for the lag between the buffer reservoir and the superfusion chamber.

Drug suppliers

5-Hydroxy[G-^{3}H]tryptamine creatinine sulphate was purchased from Amersham International plc. All other supplies were purchased from Sigma Chemical Co. with the exception of paroxetine which was a gift from SmithKline Beecham.

Calculations and statistics

The total number of counts per chamber was summed. The percentage fractional release was calculated by dividing the counts per fraction by the total chamber counts, this process was repeated for each fraction.

Unpaired Student's t-test was used to determine significance in the non-specific uptake experiments. One-way ANOVA followed by Studentised range test was used in the uptake experiments over 24 hours. In both cases values of $P \leq 0.05$ were considered statistically significant. .

Results

Uptake studies

Pre-incubation with paroxetine significantly reduced [^{3}H]5-HT uptake into hypothalamic slices; control 77.9±14.2 nmoles [^{3}H]5-HT/mg tissue wet weight, paroxetine 23.75±4.5nmoles [^{3}H]5-HT/mg tissue wet weight (unpaired Student's t-test, $P<0.001$ n=4), see figure 8.

The differences in uptake of [^{3}H]5-HT at the four time points studied is shown in figure 9; mid light 58.3±8.4nmoles/mg tissue wet weight/30 mins, end light 101±5.2nmoles/mg tissue wet weight/30 mins, mid dark 49.9±3.4nmoles/mg tissue wet weight/30 mins and end dark 60.4±1.7nmoles/mg tissue wet weight/30 mins. There was a significant difference between uptake at end light and mid dark (One-way ANOVA, $P<0.001$ and n=4-6).

Circadian rhythm studies

Application of 5-HT (1μM) had no effect on the basal release i.e. 5mM K^+ stimulus, of [^{3}H]5-HT, see table 2 and figure 10.

Treatment	*% Fractional Release*
Basal release	1.4±0.07
Basal Release + 1μM 5-HT	1.1±0.1

Table 2. Effect of 5-HT on the basal release of tritiated 5-HT. Values in % fractional release, are expressed as mean±s.e.m. and n=1 experiment performed in quadruplicate. The data quoted were obtained 16 min after application of exogenous 5-HT.

There was no difference in the fractional release of tritiated 5-HT at the four time points because exogenously applied 5-HT did not decrease [^{3}H]5-HT release, see table 3 and figures 11 to 14.

	% Fractional Release			
Treatment	*Mid Light*	*End Light*	*Mid Dark*	*End Dark*
Basal Release	1.2±0.1	1.7±0.1	2.0±0.1	2.0±0.1
25mM K^+	11.0±0.7	11.4±0.2	12.2±0.6	11.3±0.6
25mM K^+ + 5-HT	16.6±0.9	11.2±0.4	12.5±0.2	11.5±0.6

Table 3. Release of [^{3}H]5-HT from hypothalamic slices at four time points. Values, in % fractional release, are expressed as mean±s.e.m, n=1-4 experiments performed in quadruplicate. The data quoted were obtained 16 min after application of the first concentration of exogenous 5-HT (30nM).

When the concentration of the K^+ stimulus was lowered to 15mM, and the experiment repeated, there was still no inhibition of [^{3}H]5-HT release by exogenous 5-HT, see table 3 and figure 15.

Treatment	*% Fractional Release*
Basal Release	0.8±0.05
15mM K^+	1.6±0.15
15mM K^+ + 5-HT	1.85±0.075

Table 4. Effect of 10nM and 30nM 5-HT the 15mM K^+-stimulated [^{3}H]5-HT release. Values, in % fractional release, are expressed as mean±s.e.m., n=1 experiment performed in quadruplicate. The data quoted were obtained 16 min after application of the first concentration of exogenous 5-HT.

A higher single concentration of 5-HT (1μM) was added in case previous concentrations had been too low to induce a decrease in tritiated 5-HT release in the hypothalamus, the doses having been based on those effective in the cortex. The challenge failed to inhibit [^{3}H]5-HT release, see table 5 and figure 16.

Treatment	*Fractional Release*
Basal release	1.45±0.1
25mM K^+	11.5±0.8
25mM K^+ + 1μM 5-HT	11.8±0.4

Table 5. Tissue response to 1μM 5-HT. Values, in % fractional release, are expressed as mean±s.e.m., n=1 experiment performed in quadruplicate. The data quoted were obtained 16 min after application of exogenous 5-HT.

Finally the incubation time was decreased to 15 min, the tissues were stimulated with 20mM K^+ and one concentration of 5-HT (10μM) added; there was no suppression of 5-HT release, see table 6 and figure 17.

Treatment	*% Fractional Release*
Basal Release	1.7±0.07
20mM K^+	6.7±0.4
20mM K^+ + 5-HT	7.1±0.02

Table 6. Effect of a reduction in incubation time on [^{3}H]5-HT release. Values, in % fractional release, are expressed as mean±s.e.m., n=1 performed in quadruplicate. The data quoted were obtained 16 min after application of exogenous 5-HT.

Calcium studies

Removal of Ca^{++} from the perfusing medium did not inhibit the 25mM K^+-stimulated release of [^{3}H]5-HT. When the K^+ stimulus was reduced to 20mM K^+, and the experiment repeated, there was still no decrease in tritiated 5-HT output, see table 7 and figures 18 and 19 respectively.

Treatment	*% Fractional Release*
Basal Release	1.5±0.01
25mM K^+	2.7±0.25
25mM K^+ without Ca^{++}	4.0±0.2
Basal Release	0.7±0.01
20mM K^+	5.2±0.07
20mM K^+ without Ca^{++}	3.0±0.2

Table 7. Effect of calcium ion removal on the 25mM K^+- or 20mM K^+-stimulated release of [^{3}H]5-HT. Values, in % fractional release, are expressed as mean±s.e.m., n=2-3 experiments performed in quadruplicate. The data quoted were obtained 16 minutes after removal of Ca^{++} ions from the superfusing Krebs.

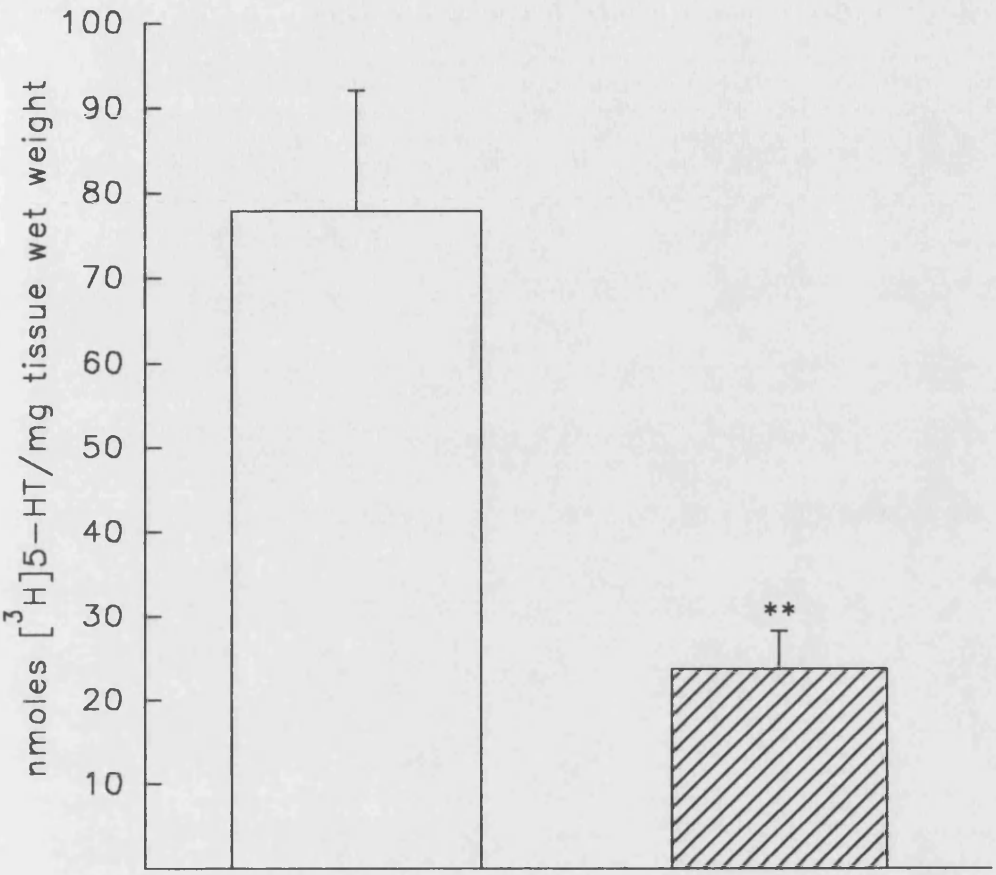


Figure 8. Hypothalamic slices were incubated with paroxetine to determine the non-specific uptake of [^{3}H]5-HT. Values, in nmoles [^{3}H]5-HT/mg tissue wet weight/15 mins, are expressed as mean±s.e.m., **P<0.001 vs control, n=4 for each treatment group. Control □ and in the presence of paroxetine ▨.

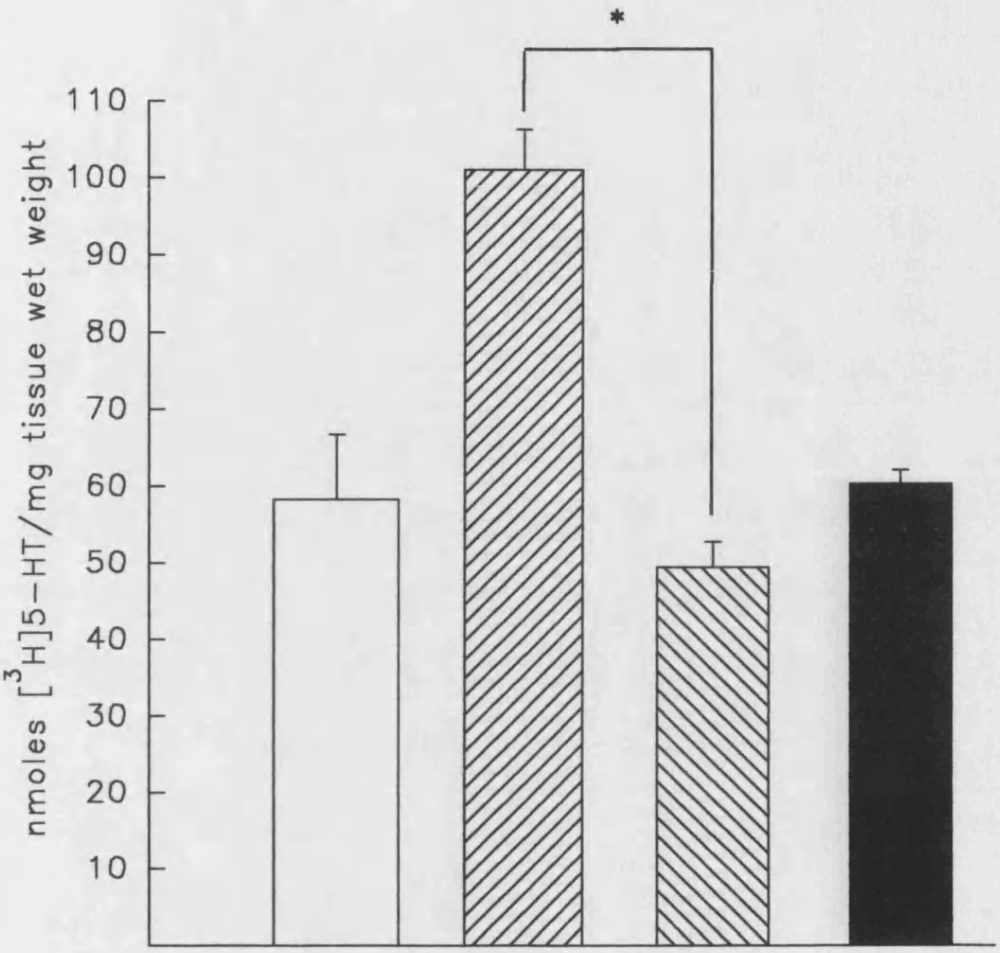


Figure 9. The uptake of [^{3}H]5-HT into hypothalamic slices was determined at mid light, end light, mid dark and end dark. Values, in nmoles [^{3}H]5-HT/mg tissue wet weight/30 min, are expressed as mean±s.e.m., *P<0.05 end light vs mid dark, n=4-6 depending on time point and treatment. Mid light □, end light ▨, mid dark ▧ and end dark ■.

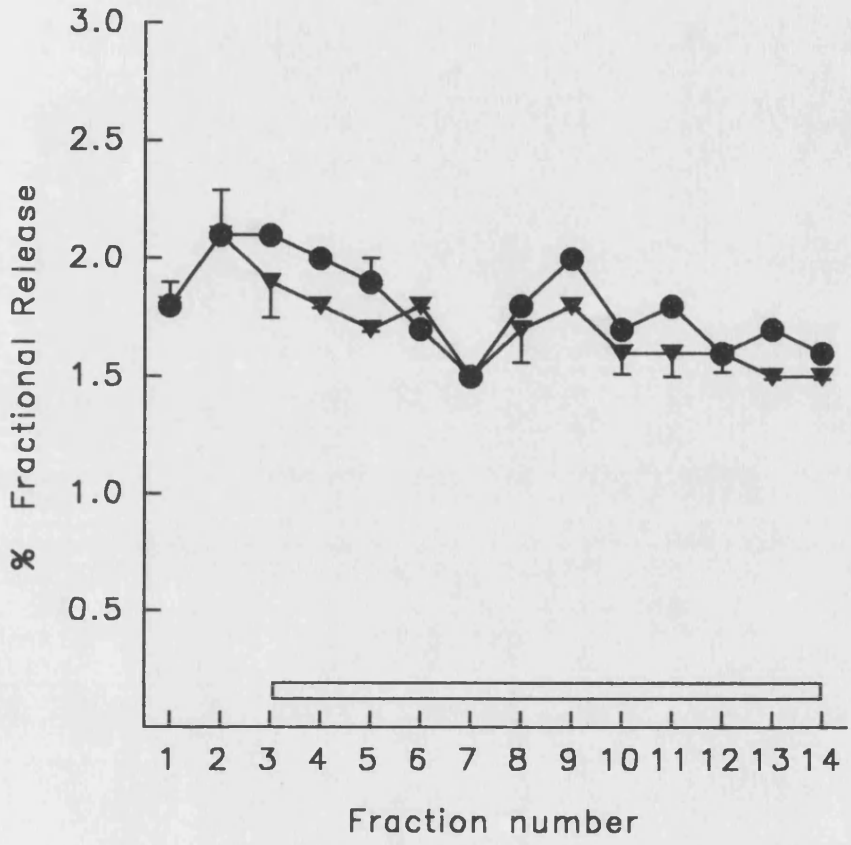


Figure 10. Effect of 1μM 5-HT on basal (5mM K^+) [^{3}H]5-HT release from hypothalamic slices. Exogenous 5-HT was present in the buffer as shown by the open bar. Values, in percentage fractional release, are expressed as mean±s.e.m., n=1 experiment performed in quadruplicate, 5mM K^+ (●) and 5mM K^+ + 1μM 5-HT (▼).

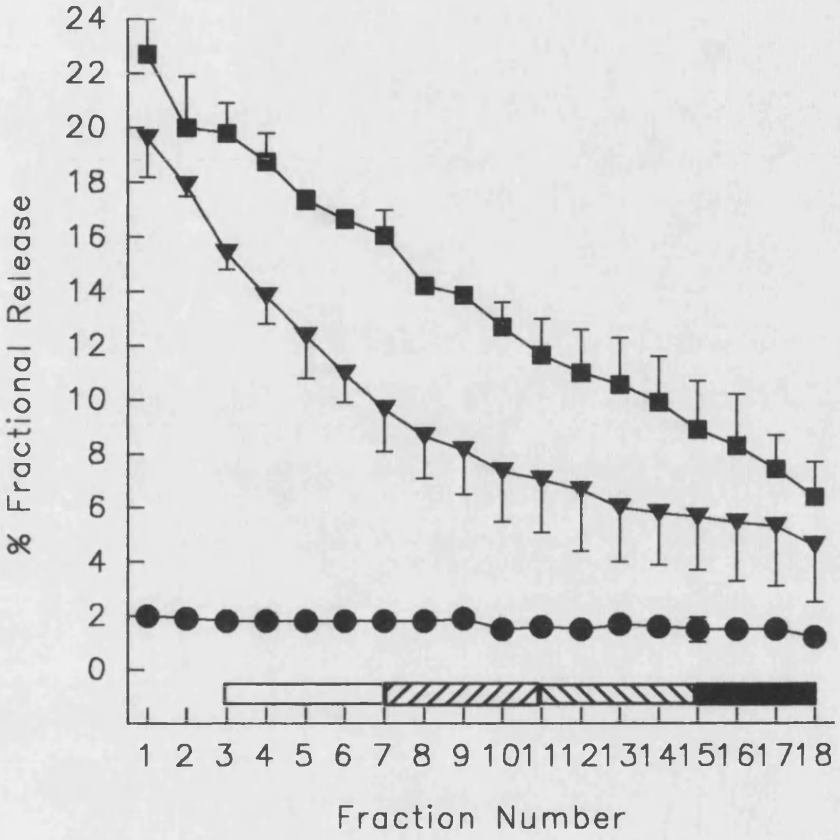


Figure 11. Effect of the addition of 5-HT (30-1000nM) on the 25mM K^+-induced [^{3}H]5-HT overflow from hypothalamic slices at **mid light**. Increasing concentrations of 5-HT were added as indicated by the bar fill; 30nM open bar, 100nM left-sloping hatched bar, 300nM right-sloping hatched bar, 1000nM solid bar. Values, in percentage fractional release, are expressed as mean±s.e.m., n=1 experiment performed in quadruplicate. 5mM K^+ (●), 25mM K^+ (▼) and 25mM K^+ + 5-HT (■).

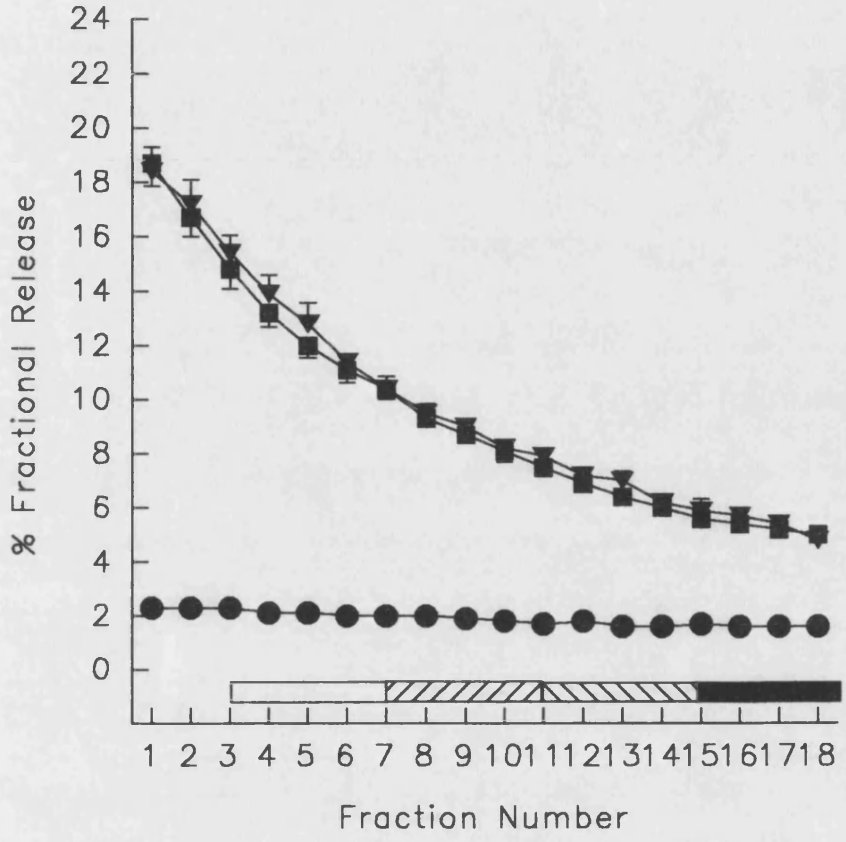


Figure 12. Effect of the addition of 5-HT (30-1000nM) on the 25mM K^+-induced [^{3}H]5-HT overflow from hypothalamic slices at **end light**. Increasing concentrations of 5-HT were added as indicated by the bar fill; 30nM open bar, 100nM left-sloping hatched bar, 300nM right-sloping hatched bar, 1000nM solid bar. Values, in percentage fractional release, are expressed as mean±s.e.m., n=3 experiments performed in quadruplicate. 5mM K^+ (●), 25mM K^+ (▼) and 25mM K^+ + 5-HT (■).

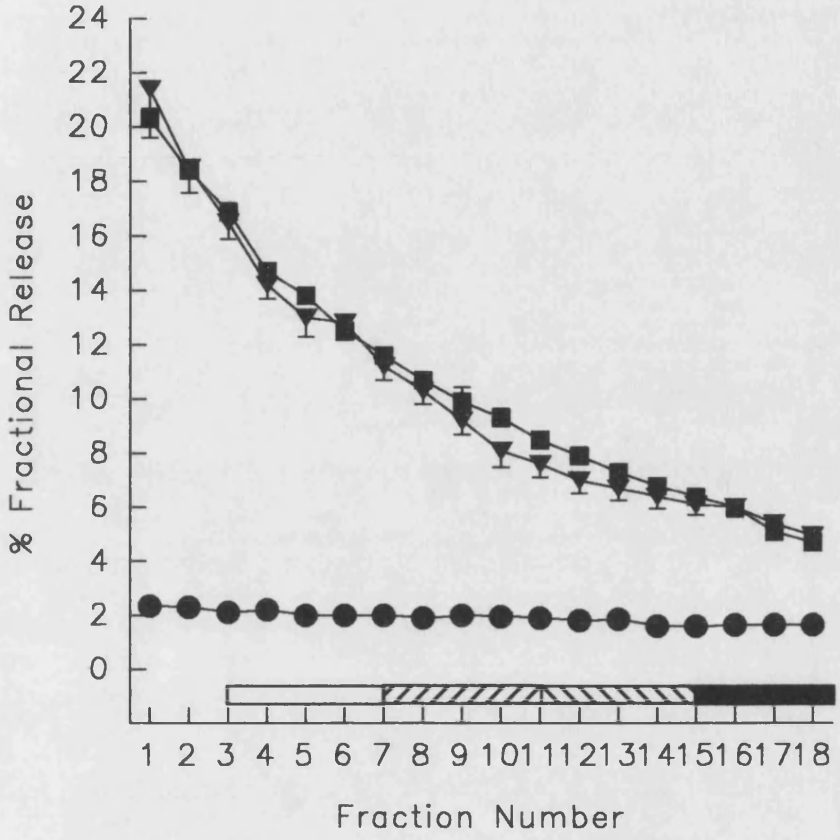


Figure 13. Effect of the addition of increasing concentrations of 5-HT (30-1000nM) on the 25mM K^+-induced [^{3}H]5-HT overflow from hypothalamic slices at **mid dark**. Increasing concentrations of 5-HT were added as indicated by the bar fill; 30nM open bar, 100nM left-sloping hatched bar, 300nM right-sloping hatched bar, 1000nM solid bar. Values, in percentage fractional release, are expressed as mean±s.e.m., n=4 experiments performed in quadruplicate, 5mM K^+ (●), 25mM K^+ (▼) and 25mM K^+ + 5-HT (■).

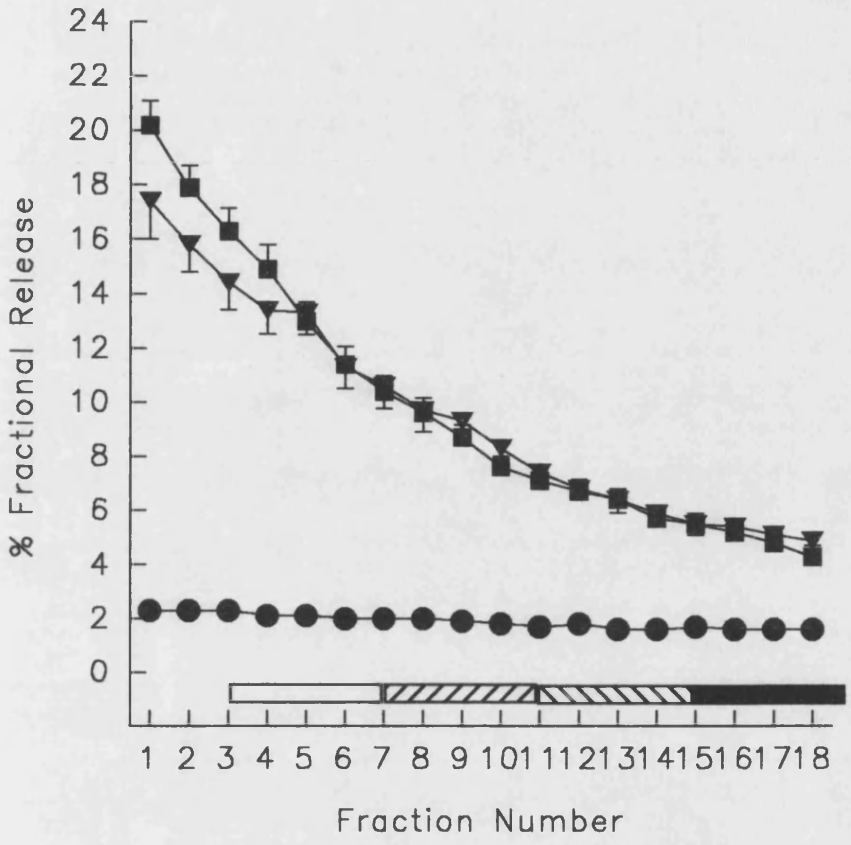


Figure 14. Effect of the addition of 5-HT (30-1000nM) on the 25mM K^+-induced [^{3}H]5-HT overflow from hypothalamic slices at **end dark**. Increasing concentrations of 5-HT were added as indicated by the bar fill; 30nM open bar, 100nM left-sloping hatched bar, 300nM right-sloping hatched bar, 1000nM solid bar. Values, in percentage fractional release, are expressed as mean±s.e.m., n=4 experiments performed in quadruplicate, 5mM K^+ (●), 25mM K^+ (▼)and 25mM K^+ + 5-HT (■).

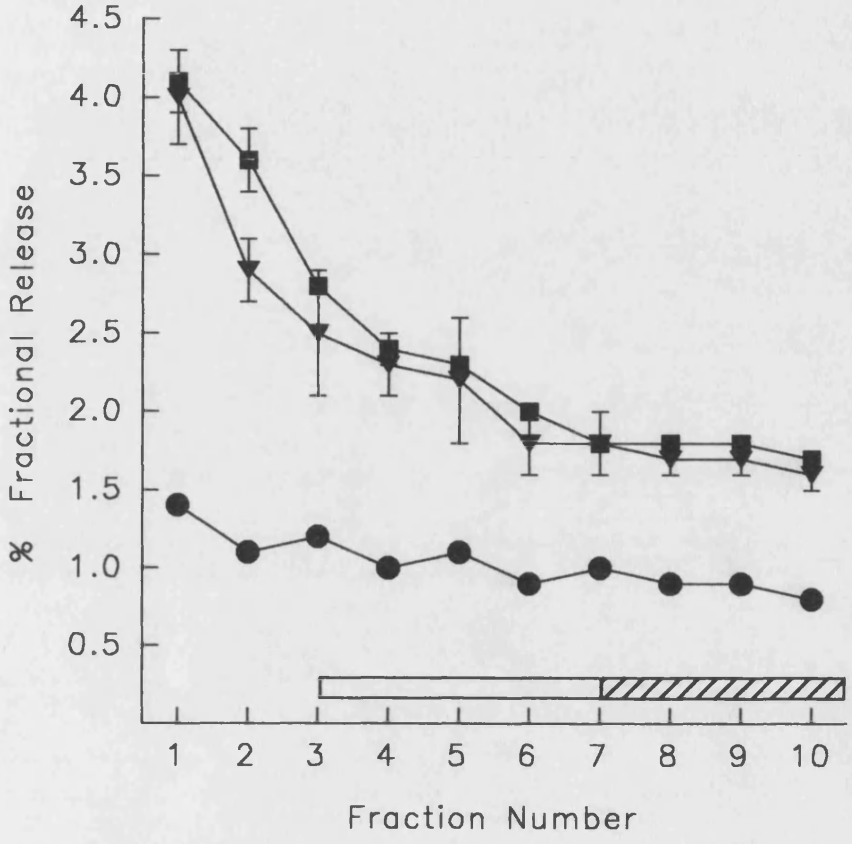


Figure 15. Effect of 10nM and 30nM 5-HT on the 15mM K^+-induced release of [^{3}H]5-HT. 5-HT was applied as indicated by the bar fill; 10nM open bar, 30nM hatched bar. Values, in percentage fractional release, are expressed as mean±s.e.m., n=1 experiment performed in quadruplicate, 5mM K^+ (●), 15mM K^+ (▼) and 15mM K^+ + 5-HT (■).

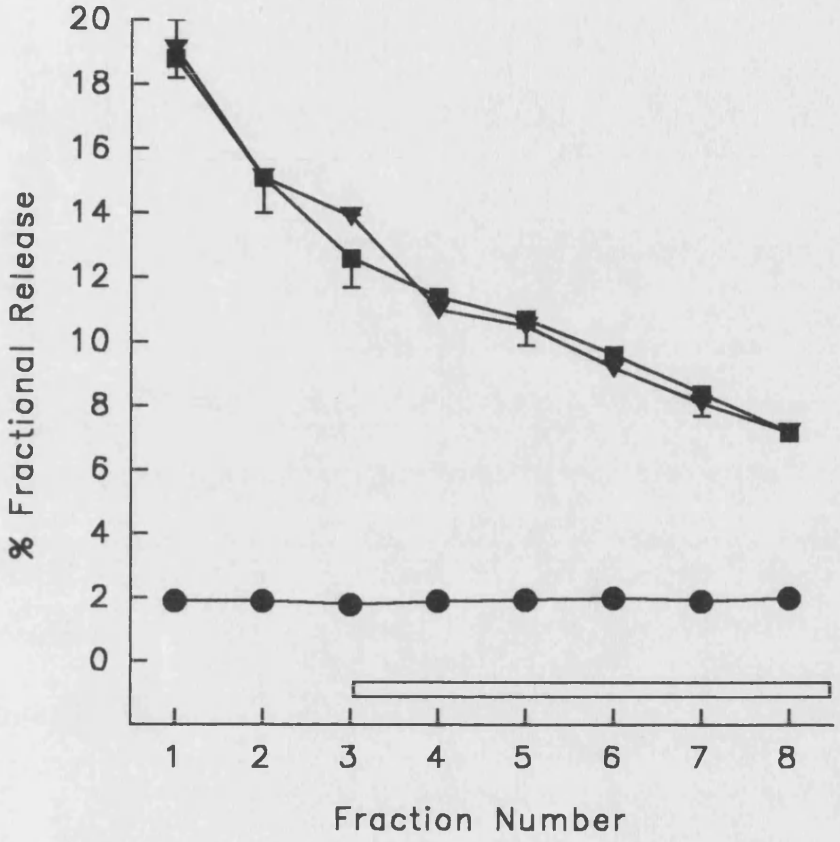


Figure 16. Effect of adding one high concentration of 5-HT (1μM), shown by the open bar, on the 25mM K^+-stimulated overflow of [^{3}H]5-HT. Values, in percentage fractional release, are expressed as mean±s.e.m., n=1 experiment performed in quadruplicate. 5mM K^+ (●), 25mM K^+ (▼) and 25mM K^+ + 1μM 5-HT (■).

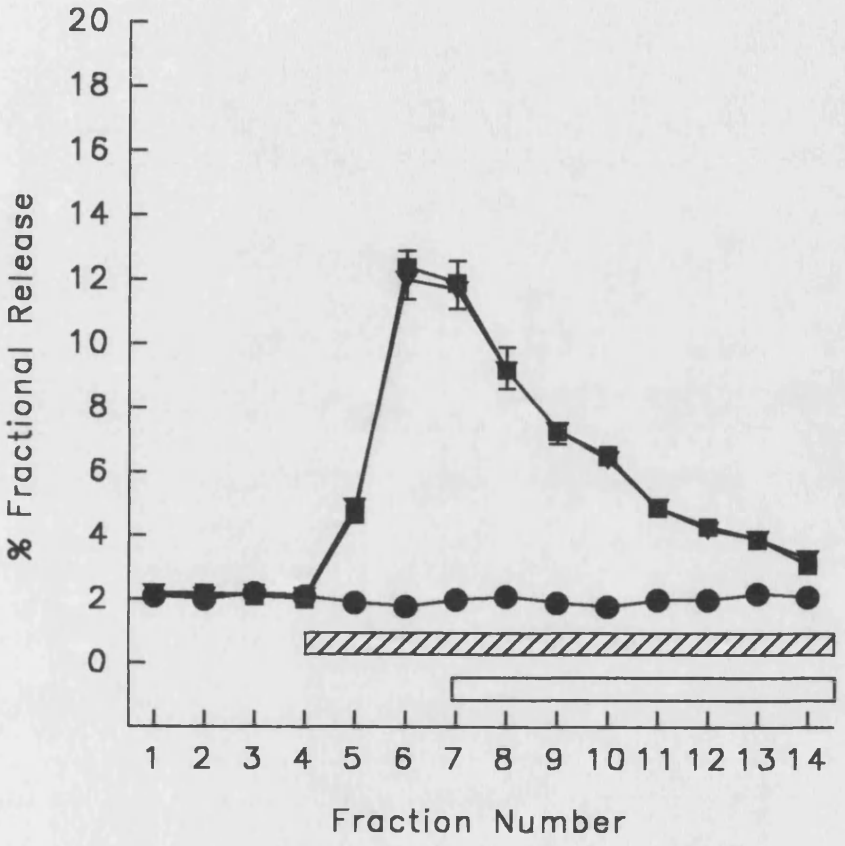


Figure 17. The incubation time was reduced to 15 minutes and the effect of 10μM 5-HT, open bar, on the 20mM K^+-stimulated release, hatched bar, assessed. Values, in percentage fractional release, are expressed as mean±s.e.m., n=1 experiment performed in quadruplicate, 5mM K^+ (●), 20mM K^+ (▼) and 20mM K^+ + 10μM 5-HT (■).

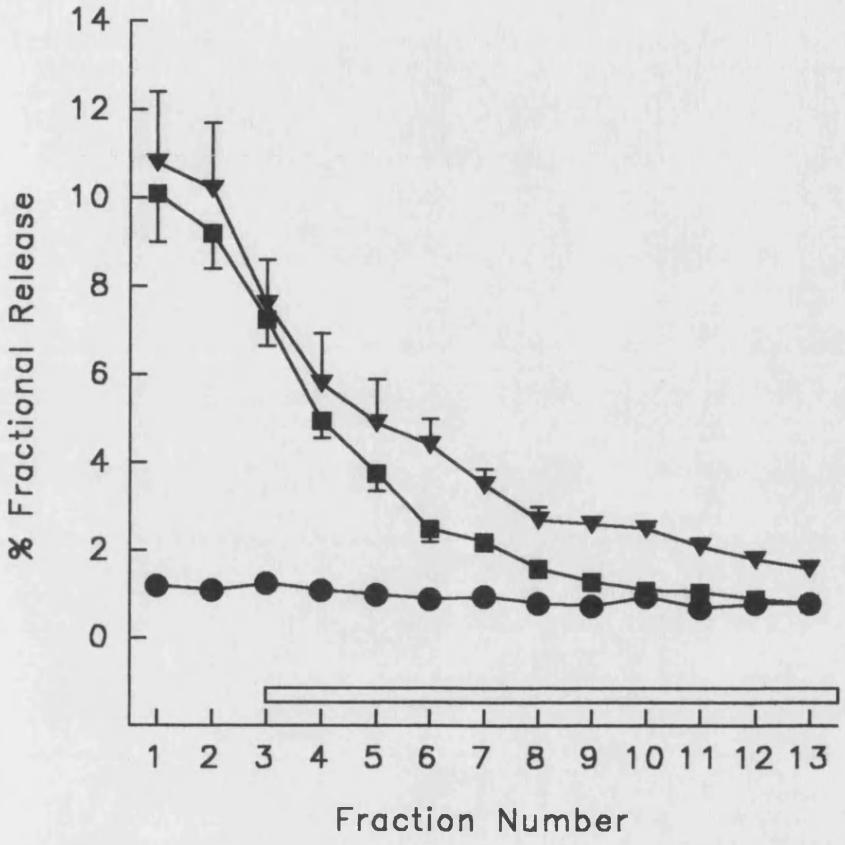


Figure 18. Calcium ions were removed from the 20mM K^+ superfusing medium, as indicated by the open bar. Values, in percentage fractional release, are expressed as mean±s.e.m., n=2-3 experiments performed in quadruplicate, 5mM K^+ (●), 20mM K^+ (▼) and 20mM K^+ without Ca^{2+} (■).

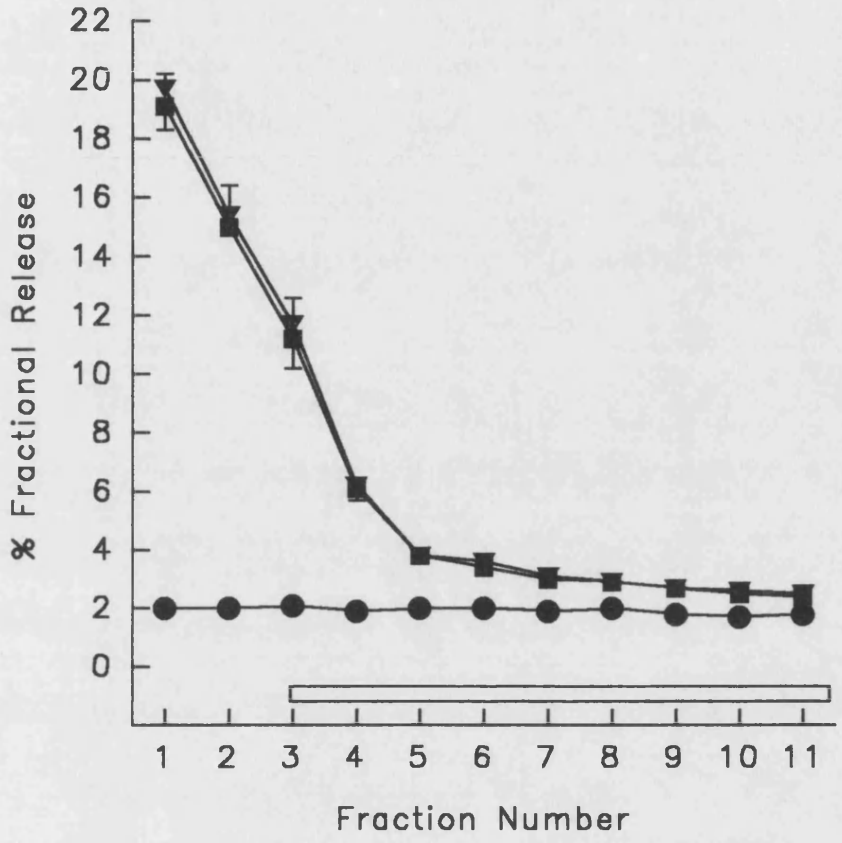


Figure 19. Calcium ions were removed from the 25mM K^+ superfusing medium, as indicated by the open bar. Values, in percentage fractional release, are expressed as mean±s.e.m., n=2-3 experiments performed in quadruplicate. 5mM K^+ (●), 25mM K^+ (▼) and 25mM K^+ without Ca^{2+} (■).

Discussion

The superfusion technique developed by Frankhuyzen and Mulder (1982), adapted to measure 5-HT release by Middlemiss (1984; 1985) and employed in our laboratory by Singh and Redfern (1994a,b) to study autoreceptor function in the hippocampus and cortex *in vitro* was applied directly to the hypothalamus.

Uptake experiments

Initial uptake experiments indicated that there was almost twice as much non-specific uptake in the hypothalamus (29.5%) as in the cortex (cortex 16%; Singh and Redfern 1994a). The uptake of tritiated 5-HT into hypothalamic slices at four time points showed a significant variation, with the peak occurring in animals sacrificed at end light, and no significant difference between the amount of [^{3}H]5-HT taken up at the other three times. However the degree of non-specific uptake was not measured at each of the time points. All these experiments were carried out within 4 days of the arrival of the same batch of [^{3}H]5-HT using randomly assigned animals. The degradation of [^{3}H]5-HT is considered to be 2% per month when stored at +2°C. In the laboratory [^{3}H]5-HT was aliquoted in 0.2mM ascorbic acid and stored at -20°C until use. It is unlikely, therefore, that the differences seen are due to degradation of the radiolabel. The results of the [^{3}H]5-HT uptake experiments are partially in agreement with Meyer and Quay (1976). These workers showed that the uptake of [^{3}H]5-HT into hypothalamic homogenates or SCN slices displayed a marked variation, with peak uptake occurring towards the end of the light phase (Meyer and Quay 1976). However the trough in uptake appeared during the earlier hours of the light cycle in both hypothalamic homogenates and SCN slices, whereas in this study the uptake into hypothalamic chips was equal at mid dark, end dark and mid light. The difference could be due to the use of basal hypothalamus; all the hypothalamic nuclei dissected could display rhythms in uptake with varying, and perhaps opposed, acrophases and barthyphases. It might have been advisable to use only one hypothalamic nucleus, but this would have entailed an increased number of rats.

24 Hour Rhythm Studies- What went wrong?

This set of experiments attempted to measure the IC_{50} value for the inhibition of [^{3}H]5-HT release by exogenous 5-HT at mid light, end light, mid dark and end dark. Thus the actual decrease in 5-HT with time was overlooked until it was found that the IC_{50} at each time point was the same. In order to check whether this was a valid observation, the time course of the experiment was plotted and showed that in fact exogenously applied 5-HT was failing to inhibit [^{3}H]5-HT release. This could not be because the exogenous 5-HT was being degraded since pargyline was present in the perfusing buffer throughout the experiment. Neither could the exogenous 5-HT be taken up into 5-HT terminals since the selective 5-HT uptake inhibitor paroxetine was present throughout the experiment at a concentration known to effective (Middlemiss 1984; 1985).

One possibility was that the 5-HT might not be reaching receptor sites because of the buffer flow round the tissues. However, since Singh and Redfern (1994a) had used the same apparatus, tissue volume and size successfully this seems unlikely. To examine the possibility that, in the hypothalamus, the initial 5-HT challenge might be too low, a high concentration of 5-HT was applied; this too failed to inhibit tritiated [^{3}H]5-HT release. It is unlikely that the 5-HT receptor was already occupied since the tissue had a 30 minute stabilisation period to allow for dissociation of ligands from receptors. A further possibility was that the concentration of radiolabel used was insufficient to label intraneuronal 5-HT stores effectively in 5-HT neurons. This is improbable since it has been shown that the uptake of 0.1μM [^{3}H]5-HT into hypothalamic slices reaches a plateau at a tissue concentration of 10mg/ml wet weight (Shaskan and Snyder 1970); the tissue concentration used in the experiments described here was about 15mg/ml. On the other hand the concentration of radiolabel may have been too high so that it entered other neurons and/or glial cells. Again this is unlikely. At a concentration of 0.1μM [^{3}H]5-HT has been shown to be preferentially taken up by serotoninergic neurons (Shaskan and Snyder 1970). The uptake of [^{3}H]5-HT into hypothalamic slices was found to have two components; a high affinity uptake

component with a K_m for 5-HT of 0.17μM and a low affinity component with a K_m for 5-HT of 80μM. At low concentrations of 5-HT the high affinity uptake system accounts for 4 times the uptake by the low affinity system (Shaskan and Snyder 1970). The incubation time may have been insufficient or too long. The uptake of [^{3}H]5-HT into hypothalamic slices reaches a plateau at an incubation time of 24 minutes (Shaskan and Snyder 1970) and reaches equilibrium in synaptosomes after 30 minutes (Wolf and Kuhn 1986). Thus the conditions used should have ensured preferential uptake into 5-HT neurons. It is possible that [^{3}H]5-HT may have been taken up into 5-HT neurons but not entered part of the functional cytoplasmic pool.

The results of the Ca^{++}-removal experiments suggest that the [^{3}H]5-HT was being taken up into glial cells since [^{3}H]5-HT release from neurons in the cortex and hippocampus has been shown to be calcium-dependent (Gothert 1980; Singh and Redfern 1994a).

There are two other possibilities for the lack of effect of 5-HT. Firstly the concentration of 5-HT used could have been too low to preferentially activate 5-HT_{1B} autoreceptors. However the IC_{50} for 5-HT inhibition of [^{3}H]5-HT using this technique, but using cortical tissue, is 46nM (Middlemiss 1984) and 1μM 5-HT had no effect in this study. 5-HT_3 receptors have been demonstrated to affect 5-HT release in the ventral hippocampus (Martin et al. 1992). Stimulation of these receptors increases 5-HT release since the 5-HT_3 receptor is a cation channel. The relative K_i values of 5-HT for 5-HT_{1B} and 5-HT_3, receptors derived from binding studies, are 7.6nM (Peroutka 1986) and 270nM (Maricq et al. 1991) respectively. Thus the 5-HT_{1B} receptor would have been preferentially stimulated at the concentrations of 5-HT (30nM-1μM) used in this study. Additionally there are few 5-HT_3 receptors in the hypothalamus (Palacios et al. 1990). An alternative explanation is that the K^+ stimulus might stimulate the release of other neurotransmitters which might enhance 5-HT release *e.g.* nicotinic acetylcholine receptors (Hery et al. 1977a). However, since [^{3}H]5-HT release was not calcium-dependent it would argue against this factor. An uptake inhibitor was included in the superfusing buffer so exogenous 5-HT could not

enter 5-HT neurons (and perhaps glia) and displace [^{3}H]5-HT intracellularly. It is also unlikely that 5-HT displaced [^{3}H]5-HT from, for example dopamine neurons, since no increase in [^{3}H]5-HT was seen even after the maximal concentration of 5-HT.

The hypothalamus is rich in glial cells which are known to possess two amine uptake systems. The first is a low affinity system which is temperature and sodium ion independent, with a K_m for 5-HT of 1.5mM. The second system has a high affinity for 5-HT, comparable to that of neurons, and is temperature and sodium ion dependent, it has a K_m of 0.4µM for 5-HT (Katz and Kimberger 1985). This second system is also susceptible to inhibition by uptake inhibitors such as clomipramine (Suddith et al. 1978). Thus the results showing 29.5% non-specific uptake would probably not have included this high affinity uptake site, indeed the degree of non-specific uptake could even be higher.

In order to determine whether the results obtained arose because the technique was inappropriate for hypothalamic tissue, additional experiments were performed. The stimulation was changed to two pulses of 25mM K^+, each 4 minutes long, the first (S_1) 8 minutes after the end of the stabilisation period and the second (S_2) 40 minutes later. Drugs to be tested were added 20min before S_2 and the ratio of S_1/S_2 calculated. Stimulation produced a good increase in [^{3}H]5-HT output over basal fractional release, however exogenously applied 5-HT (1µM) failed to inhibit 25mM K^+-induced [^{3}H]5-HT output and had no effect on basal [^{3}H]5-HT fractional release. Electrical stimulation of the tissues was attempted using two different commercially available superfusion systems, one from Linton Instrumentation, see figure 20, and a Brandel superfusion system. In the first system no current flow was detected at low voltages. At high voltages (150V) the platinum electrodes oxidised but there was still no current flow. In the second system current flowed between the electrodes but there was never a reproducible release of [^{3}H]5-HT. Two stimulation parameters were tried. The first is designed to prevent build up of [^{3}H]5-HT around the tissue on stimulation which might inhibit further [^{3}H]5-HT output (Fischer et al. 1990; O'Connor and Kruk 1991). Pulses were delivered at a high frequency (100Hz) for a very short period (10

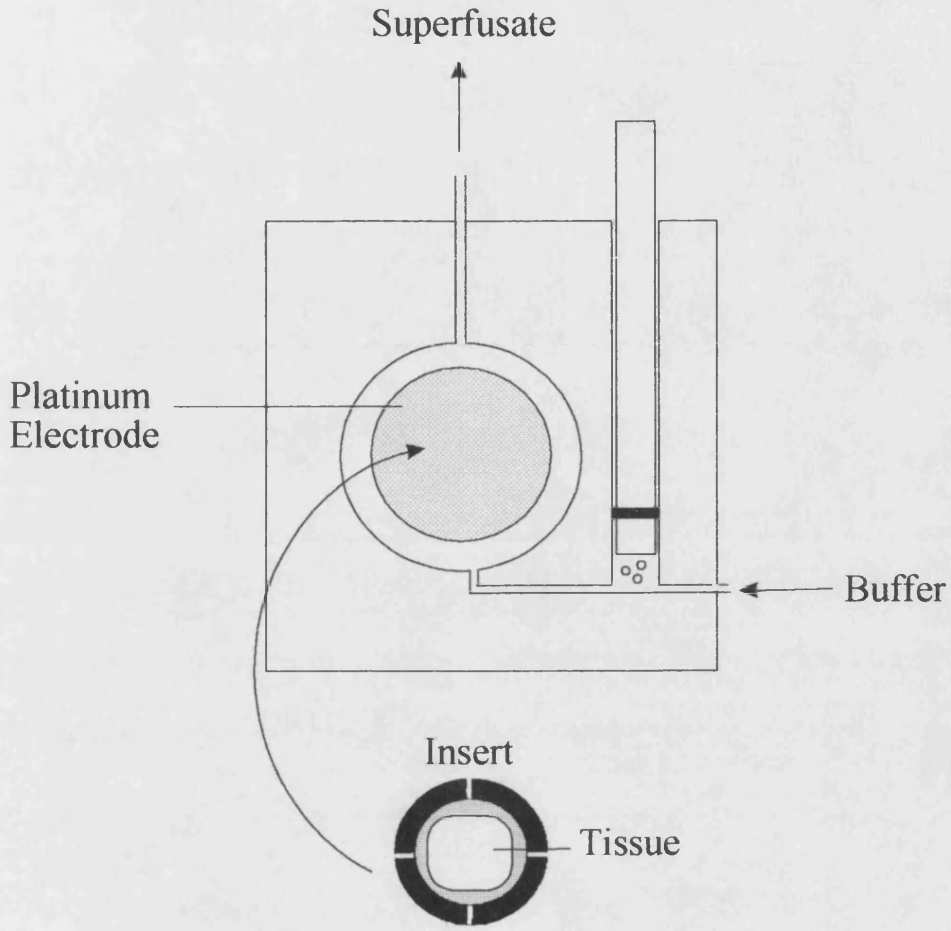


Figure 20. Cross section through the Linton Instrumentation superfusion apparatus used. Tissue is placed on a nylon mesh and inserted into the well of the chamber, which lies in one half of the block. The two halves of the superfusion block are then screwed together and the whole inserted into a water bath maintained at 37°C. Buffer flows in at a side inlet, over the tissue chips and out the top of the superfusion block. Air bubbles can be removed by a siphon device which opens into the chamber. A platinum electrode is built into each half of the superfusion block to stimulate the tissue.

sec). The second was a more physiological approach using the same frequency as 5-HT neuronal firing during quiet waking (3-5Hz) (Jacobs and Azmitia 1992), but for a long duration (4 min). Neither stimulation parameter produced a reproducible release of [^{3}H]5-HT.

Binding sites for 5-HT have been demonstrated on horse striatal astroglia (Murphy and Pearce 1987). There is debate whether these sort of receptors are functionally linked; some reports have shown that the receptors are positively linked to adenylate cyclase. It might be thought that glial cells cannot be depolarised by K^+, but one study demonstrated the K^+-induced release of GABA from glia (Murphy and Pearce 1987). 5-HT stimulation of glia can also lead to the release of taurine, a highly active neuropeptide, which increases chloride conductance and would therefore hyperpolarise neurons. Thus [^{3}H]5-HT taken up into glia might be released uncontrollably by perfusion of a medium containing elevated K^+.

The data here are in agreement with the findings of Passarelli and colleagues (1987) which suggest that the hypothalamus is different from other brain regions. These workers compared electrically- and 20mM K^+ pulse-stimulated release of [^{3}H]5-HT in hypothalamic slices and found significant differences. When calcium ions were removed from the superfusing buffer 20 min prior to electrical stimulation the release of [^{3}H]5-HT was almost entirely prevented; this was not true of K^+-stimulation. If slices were superfused for 40 min in the presence of the calcium ion chelator EGTA (1mM), [^{3}H]5-HT release was only inhibited by 40-50%. The effects of tetrodotoxin varied with stimulus. Electrically-evoked [^{3}H]5-HT output was inhibited by tetrodotoxin, but if elevated K^+ was used as the stimulus the output was unaffected by tetrodotoxin. In addition the paper compared the effects of lysergic acid diethylamine (LSD), 5-methoxytryptamine (both autoreceptor agonists) and methiothepin on electrically and K^+-evoked tritiated 5-HT release. The [^{3}H]5-HT-inhibiting effects of the agonists were markedly attenuated when elevated K^+ was used as the depolarising stimulus. These workers concluded that the [^{3}H]5-HT output observed in the hypothalamus was either from astrocytes or a non-functional cytoplasmic pool in

serotoninergic neurons.

Summary

Using the technique of *in vitro* superfusion, an attempt was made to measure the sensitivity of the terminal 5-HT autoreceptor in hypothalamic slices at four, equally spaced time points through the light-dark cycle. The hypothalamus appears to behave differently from the hippocampus and cortex *in vitro*. The results indicate that radiolabelled 5-HT is actively taken up into hypothalamic glial cells where its release can not be controlled. One way to overcome the problem could be to measure [^{3}H]5-HT release from purified hypothalamic synaptosomes. The technique could have been verified by trying to repeat experiments in the cortex or by using a 5-HT_{1B} receptor agonist, such as 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridyl)-1H-indole (RU24969) instead of 5-HT. However, using the same technique, Middlemiss (1985) found that the IC_{50} for RU24969 was 35nM in cortical slices which is not different from the IC_{50} for 5-HT (46nM). Since the technique proved to be unviable, no conclusions can be drawn with regard to the hypothesis of this section.

Chapter 3 5-Hydroxytryptophan Accumulation

Hypothesis

Synthesis was the second parameter of 5-HT function to be studied. The experiments in this chapter investigated the hypotheses that, in entrained rats;

a) The degree of 5-HT_{1B}-mediated inhibition of 5-HT synthesis varies significantly over 24 hours.

b) Chronic antidepressant treatment would down-regulate this 5-HT_{1B}-mediated control.

c) The scale of down-regulation would be significantly different over the light-dark cycle.

The experiments were divided into three sections; the first section aimed to confirm previous demonstrations of a rhythm in basal 5-HT synthesis, the second section to verify whether 5-HT synthesis could be affected by RU24969, a 5-HT_{1B} agonist, and thus pharmacologically define the receptor mediating the effect; the final section investigated the effect of chronic antidepressant treatment on RU24969-mediated control of 5-HT synthesis.

Four time points spaced equally through the light-dark cycle were chosen; these were mid light (Circadian Time 6), end light (CT11.45), mid dark (CT 18) and end dark (CT23.45). Circadian time refers to the time in hours after lights on in the relavent animal colony. These time points were chosen because, from previous work, they should coincide with maxima and minima in enzyme activity so that comparisons could be made with the literature.

Method

Animals

Groups of 6 phase-shifted male Wistar rats, as described on page 39, weighing 230-270g were used throughout the experiments. In each group, 2 rats served as controls, receiving the drug vehicle; control rats received the same injection volume,

and the same number of injections at corresponding time intervals as experimental rats. The remaining rats were drug treated. Treatments were not randomised, i.e. the rats in the group that were to receive treatment were all injected with the same dose of the same drug e.g. RU24969. Similar treatments were performed within a week so that, for example, groups of rats used for RU24969 (3 or 9mg/kg i.p.) studies were treated, killed and the tissue samples processed/frozen within 5 days of each other. This protocol was adopted for several reasons. First there was a limit both to the total number of rats available at any one time and to the number of rats that could be phase-shifted together. Secondly, randomisation of drug treatments might not take into account circannual rhythms in 5-HT receptors which have been demonstrated (Weiner et al. 1992). Third it ensured that control rats and treated rats had been subjected to the same environmental conditions and finally it reduced the risk of differences in breeding. Control rats were included in every set of drug treatments at every time point to cross-check 5-HTP levels with other controls and basal levels. If the manipulations were performed during the rats' light phase the animals were injected under normal room lighting, however rats injected during their dark phase were treated in dim red light (4-6 lux). Rats were subjected to either of the two protocols below. Each stage of the protocol was separated by 30 minutes.

Agonist → NSD 1015 → cervical dislocation at appropriate phase of light-dark cycle.

Antagonist → agonist → NSD 1015 → cervical dislocation at appropriate phase of the light-dark cycle.

After each stage of the treatment animals were returned to their cages and the cage placed back inside the appropriate box of the environmental cabinet. Doses of drugs and the timing between injections were assessed from published work.

Tissue preparation

Rats were killed by cervical dislocation and the whole brain removed from which the frontal cortex, hippocampus, hypothalamus and striatum were dissected onto

ice. These particular brain regions were chosen since their innervation from the RN differs; the hypothalamus and hippocampus receive afferents both the MRN and DRN whilst the input to the frontal cortex is solely from the MRN and that to the striatum from the DRN. The brain regions were weighed and the frontal cortex and hippocampus wrapped in foil and frozen at -75°C until assay. Hypothalamic and striatal tissue was homogenised in 10% w/v 0.1M perchloric acid (Tri-R Stir-R) at 4°C. The homogenates were ultracentrifuged at 15,000 x g for 15 min at 4°C (Beckman preparative ultracentrifuge, model LM-8). Supernatants were filtered through 0.45µm nylon acrodiscs (Gelman Sciences, Fisons), aliquoted into amber light-proof microcentrifuge tubes (Eppendorf, BDH) and the supernatants frozen at -20°C until they were assayed for their 5-HTP content by reverse phase high performance liquid chromatography coupled to electrochemical detection (HPLC-ECD). The pellets were re-suspended in Tris-HEPES buffer (composition in mM: NaCl 118; KCl 4.8; $MgSO_4$ 1.2; $CaCl_2$ 2.5; HEPES 20; Tris HCl 200; containing 0.1% Triton X-100) and assayed for their protein content. Microprotein assays were performed using BioRad protein dye reagent with bovine serum albumin serving as the standard.

Measurement of 5-HTP in the supernatant

An HPLC system to measure 5-HTP levels had been set up and optimised previously in the laboratory. However the system has been dissembled and it was therefore necessary to establish and optimise a new system. Separation of 5-HTP, 5-HT and 5-HIAA had been achieved in 30 minutes in the former system with the following parameters; electrode potential 0.6V, flow rate 1ml/min, octane sulphonic acid 0.3mM, EDTA 0.1mM, NaH_2PO_4 0.07M, 8% methanol v/v adjusted to pH 2.75, with the column kept at 30°C by a water bath.

The parameters for resolution of l-DOPA, DA, 5-HTP, 5-HT, 5-HIAA for the HPLC system described in this section were based on the previous optimisation, but slightly modified to produce a shorter run time (15 min). Samples (200µl) were automatically injected (Gilson sample injector, model 231 attached to a Gilson dilutor,

model 401, Anachem) via a Rheodyne sample injector (model 7125) fitted with a 100µl loop, onto a reverse phase HPLC column packed with 5µm Hypersil ODS (20cm x 5cm o.d.). The constituents of the sample were separated along the column and detected by an amperometric detector set at +0.6V (BAS LC3A amperometric detector, Anachem). The chromatogram was plotted onto a chart recorder (J.J. Lloyd Instruments). Mobile phase (composition in mM: octane sulphonic acid 0.3; EDTA 0.1; NaH_2PO_4 70; 12% v/v methanol to pH 2.75 with 6M sulphuric acid) was pumped through the system at a rate of 1ml/min by a Constametric LDC model III pump. The flow rate was lowered to 0.1ml/min when the HPLC was not in use. Fresh buffer was made up every 2-3 days as required using double-distilled, filtered water (Milli Q). The buffer was filtered under vacuum through 0.2µm nitrocellulose membrane filters (Whatman, Fisons) and bubbled with helium for 10 min to purge any dissolved oxygen from the buffer. Each month the system was flushed through with 90:10 methanol:Milli Q at a rate of 8ml/min. In addition the pump was flushed through with 0.1% warm Decon solution at 8ml/min for 10 min, then Milli Q water and finally with the methanol:Milli Q solution. A signal to noise ratio of 2:1 was considered acceptable.

Perchloric acid (0.1M) was injected onto the HPLC column to check whether it produced a peak that might interfere with the interpretation of the chromatogram. The concentration of 5-HTP in the supernatant was determined by comparing the ratio of the peak height of the internal standard (5,7-dihydroxytryptamine) to the peak height of a known concentration of 5-HTP. A standard curve was constructed from these values and the concentration of 5-HTP in the sample read from this standard curve.

The recovery of 5-HTP through the preparation stages was also checked. A known concentration of 5-HTP was prepared and stored as for brain samples. A sample of 5-HTP (200µl) prepared this way was injected onto the HPLC column, the peak height measured, and the concentration calculated as above. The recovery of 5-HTP through these stages was 94±3%.

Chronic antidepressant treatment

Rats, housed as described previously, were treated for 21 days with a once daily injection of either paroxetine hydrochloride (10mg/kg i.p.), desipramine hydrochloride (10mg/kg i.p.) or an equivalent volume of saline. In each group at least two rats served as saline-treated controls. Injections were performed once a day within an 8 hour window, to prevent the rats taking the injections as a time cue. If the rats were in their dark cycle at the time of injection then dim red light was used, as for cage cleaning. All experiments were performed after a 24 hour washout period. Antidepressant drug doses were chosen following literature review as being typical doses used. In order to assess any antidepressant-induced alterations in 5-HT_{1B} receptor function, the potency of RU24969 (9mg/kg i.p) was re-evaluated after chronic antidepressant treatment. It is known that monoamine levels and neurons decrease with age (Tatton et al. 1991), therefore rats were treated with antidepressant drugs and phase-shifted at the same time, to ensure that the rats' final weight were comparable to those of earlier studies.

Drugs

RU24969, desipramine hydrochloride and paroxetine hydrochloride were all made up in 0.9% saline. (±)Cyanopindolol and (-)n-tert-butyl-3-(4-(2-methoxyphenyl)piperazin-1-yl)-2-phenylpropionamide dihydrochloride ((+)WAY100,135) were first dissolved in a few drops of glacial acetic acid and then made up in 0.9% saline and brought to pH 7.4 with 6M sodium hydroxide. m-Hydroxybenzylhydrazine (NSD 1015) was made up in 0.9% saline and adjusted to the correct pH with 6M sodium hydroxide. In all experiments control animals received the drug vehicle.

Drug Suppliers

Drugs were purchased from the suppliers as follows; m-hydroxybenzylhydrazine (NSD 1015), desipramine hydrochloride, Triton X-100,

HEPES, Tris hydrochloride, bovine serum albumin (Sigma Chemical Company), octanesulphonic acid, NaCl, KCl, $MgSO_4$, $CaCl_2$, NaH_2PO_4 and EDTA all HPLC grade (Sigma Chemical Company); perchloric acid (BDH) and HPLC grade methanol (Fisons).

The following drugs were donated by the companies indicated in brackets, RU24969 (Roussel-Uclaf), (±)cyanopindolol (Sandoz), paroxetine hydrochloride (SmithKline Beecham) and (+)WAY100135 (Wyeth).

Statistics

Data for variations in 5-HTP concentration over 24 hours and vehicle controls vs basal levels was analysed by one-way analysis of variance (ANOVA) followed by Studentised range test. All other results were analysed using two-way ANOVA with *post hoc* Studentised range test. Values of $P<0.05$ or less were considered statistically significant.

Since the measurement of 5-HTP after studies using WAY100135 was not performed on the same HPLC system and the samples were prepared slightly differently, homogenisation in 5%w/v perchloric acid and inclusion of sodium metabisulphite in the homogenising buffer, the data have been normalised. Thus, the 5-HTP levels from treated animals were calculated as a percentage of the average 5-HTP levels of the WAY100135 vehicle control animals. The data from WAY100135 vehicle control animals was then assumed to be the average of the 5-HTP values obtained from previous vehicle control animals (statistical analysis had indicated there was no difference between basal levels and vehicle control levels of 5-HTP from rats used in RU24969 and (±)cyanopindolol studies, see later) and the 5-HTP level after WAY100135 recalculated using these new WAY100135 vehicle control 5-HTP levels. Percentages of control value were used for statistical analysis involving data derived from WAY100135 studies. Statistical methods and outcomes are set out in appendix 1.

Results

Variation in 5-HTP levels over 24 hours

Basal levels of 5-HTP, i.e. without decarboxylase inhibition, measured at mid light only were below the detection limit of the HPLC system. Following decarboxylase inhibition measurable amounts of 5-HTP were present in samples from each time point and brain region.

An example of a chromatogram obtained from a hypothalamic sample is shown in figure 21. Quantitative data from all the following studies are presented in appendix 1. The levels of 5-HTP, after decarboxylase inhibition, in the hypothalamus, hippocampus, frontal cortex and striatum are shown together in figure 22 and table A1 of appendix 1. There were significant differences between the 5-HTPacc with time in each brain region. There was a significant variation in the levels of 5-HTP measured in the hypothalamus at the four time points. In the hypothalamus the peak accumulation of 5-HTP was at mid dark (1.15±0.06ng 5-HTP/µg protein/30 mins), whilst the trough was at end dark (0.75±0.03ng 5-HTP/µg protein/30 mins), the levels at the other two time points were 1.08±0.06ng 5-HTP/µg protein/30 mins at mid light and 0.94±0.04ng 5-HTP/µg protein/30 mins at end light. The hippocampus showed a significant variation in 5-HTP levels over 24 hours. Peak 5-HTPacc occurred at mid light (6.7±0.3ng 5-HTP/µgprotein/30 mins) whereas lowest levels were determined at end dark (3.8±0.2ng 5-HTP/µg protein/30 mins); other levels were 4.65±0.2ng 5-HTP/µg protein/30 mins at end light and 5.9±0.4ng 5-HTP/µg protein/30 mins at mid dark. In the frontal cortex levels varied significantly over 24 hours, the zenith occurring at mid light (7.0±0.3ng 5-HTP/µg protein/30 mins) whilst the nadir was at end dark (3.6±0.2ng 5-HTP/µg protein/30 mins); other levels were 6.8±0.45ng 5-HTP/µg protein/30 mins at end light and 6.3±0.4ng 5-HTP/µg protein/30 mins at mid dark. The striatum showed peaks and troughs in 5-HTPacc that were directly opposite to those of the frontal cortex and hippocampus. Striatal 5-HTP levels varied significantly over the four time points. Peak 5-HTPacc was measured at end dark (7.4±0.2ng 5-HTP/µg protein/30 mins) whilst the trough was at mid light (1.7±0.09ng 5-HTP/µg protein/30

mins); other levels were 4.7±0.4ng 5-HTP/µg protein/30 mins at end light and 2.8±0.2ng 5-HTP/µg protein/30 mins at mid dark.

Vehicle controls

In no brain region and at no time point did 5-HTPacc in vehicle treated animals differ significantly from basal 5-HTPacc. Data from vehicle controls from WAY100135 studies has not been included. Statistical analysis was performed by one-way ANOVA with *post hoc* Studentised range test, with corresponding treatment vehicle controls, but, in graphs vehicle control data was pooled for simplicity. All data and statistical analysis is presented in appendix 1.

Agonist and Antagonist Studies over 24 hours

Adminstration of RU24969 (9mg/kg i.p.) caused the appearance of a marked 5-HT syndrome, but the syndrome did not appear to be more pronounced at one time point relative to another. The syndrome was not present when (±)cyanopindolol was administered before RU24969 (9mg/kg i.p.), but persisted after (+)WAY100135 pre-treatment.

Hypothalamus

RU24969 (3mg/kg i.p.) did not significantly affect 5-HTPacc at any time point. However at the higher dose of 9mg/kg i.p. RU24969 significantly decreased 5-HTPacc at all times, -27±0.9% at mid light, -27±3.6% at end light, -24±5.6% at mid dark and -35±9% at end dark. The degree of inhibition did not vary significantly over the time points though. Pre-treatment with (±)cyanopindolol (CP, 3mg/kg i.p.) blocked the RU24969-mediated decrease in 5-HTPacc, whilst having no effect on 5-HTPacc when administered alone. Interestingly, treatment with CP and RU24969 at mid light significantly increased 5-HTPacc. (+)WAY100135 (WAY, 5mg/kg i.p.) pre-administration did not block the effects of RU24969 (9mg/kg i.p.) and had no effect on 5-HTPacc when administered alone, see figure 23 and 24 and table A2 of appendix 1.

Hippocampus

At mid dark RU24969 (3mg/kg i.p.) significantly decreased 5-HTPacc by 26±0.3%. The effect at this time point was significantly different from the effects of RU24969 (3mg/kg i.p.) at the other three times. RU24969 (9mg/kg i.p.) significantly decreased 5-HTPacc at all times but the degree of inhibition varied significantly with time. CP pre-treatment blocked the 5-HTPacc-suppressing effects of RU24969 (9mg/kg i.p.). By itself CP had no effect on 5-HTPacc at mid light and mid dark but significantly increased 5-HTPacc at end light and end dark, this effect being significantly different with time. This may indicated a degree of autoinhibitory tone in the hippocampus at these time points. WAY (5mg/kg i.p.) pre-administration had no effect on the RU24969-induced suppression of 5-HTPacc and had no effect on 5-HTPacc when administered alone, see figure 25 and 26 and table A3 of appendix 1.

Frontal Cortex

RU24969 (3mg/kg i.p.) significantly decreased 5-HTPacc at end light by 15±1%. The effect of RU24969 at this time point was significantly different from its effects at the other three time points. RU24969 (9mg/kg i.p.) decreased 5-HTPacc significantly at all times, and RU24969's effects varied significantly with time. CP pre-treatment blocked the effects of RU24969, whilst having no effect when administered alone. Pre-administration of WAY did not affect the RU24969-induced decrease in 5-HTPacc and had no effect on 5-HTPacc when administered by itself, see figure 27 and 28 and table A4 of appendix 1.

Striatum

At mid light RU24969 (3mg/kg i.p.) significantly decreased 5-HTPacc by 13±3%, the effect at mid light was significantly different from the effect of RU24969 (3mg/kg i.p.) at other time points. RU24969 (9mg/kg i.p.) decreased 5-HTPacc significantly at all times, and the effect differed significantly with time. Pre-administration of CP blocked the RU24969-mediated decrease in 5-HTPacc, having no effect when administered alone. WAY pre-treatment did not affect the suppression of 5-HTPacc caused by RU24969, and had no effect when administered by itself, see

figure 29 and 30 and table A5 of appendix 1.

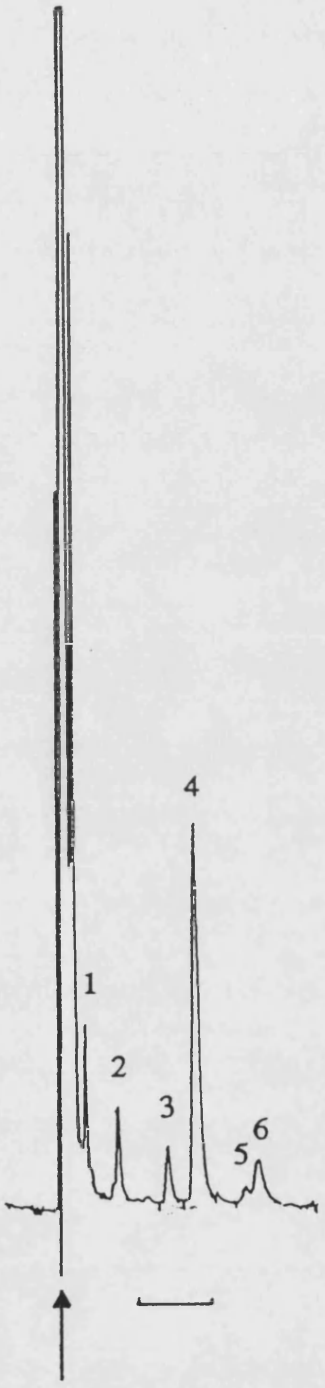


Figure 21. Example of a hypothalamic chromatogram. The arrow indicates the solvent front, l-DOPA (1) R_t 2.5 min, DA (2) R_t 4 min, 5-HTP (3) R_t 6.5 min, 5,7-DHT (4) R_t 8 min, 5-HIAA (5) R_t 10.5 min and 5-HT (5) R_t 11 mins. The detector potential was set at +0.6V and the sensitivity was 0.5nAmp full scale deflection, the bar denotes 5 mins. Details of the mobile phase and other conditions are described in full detail in the text on pages 68-69.

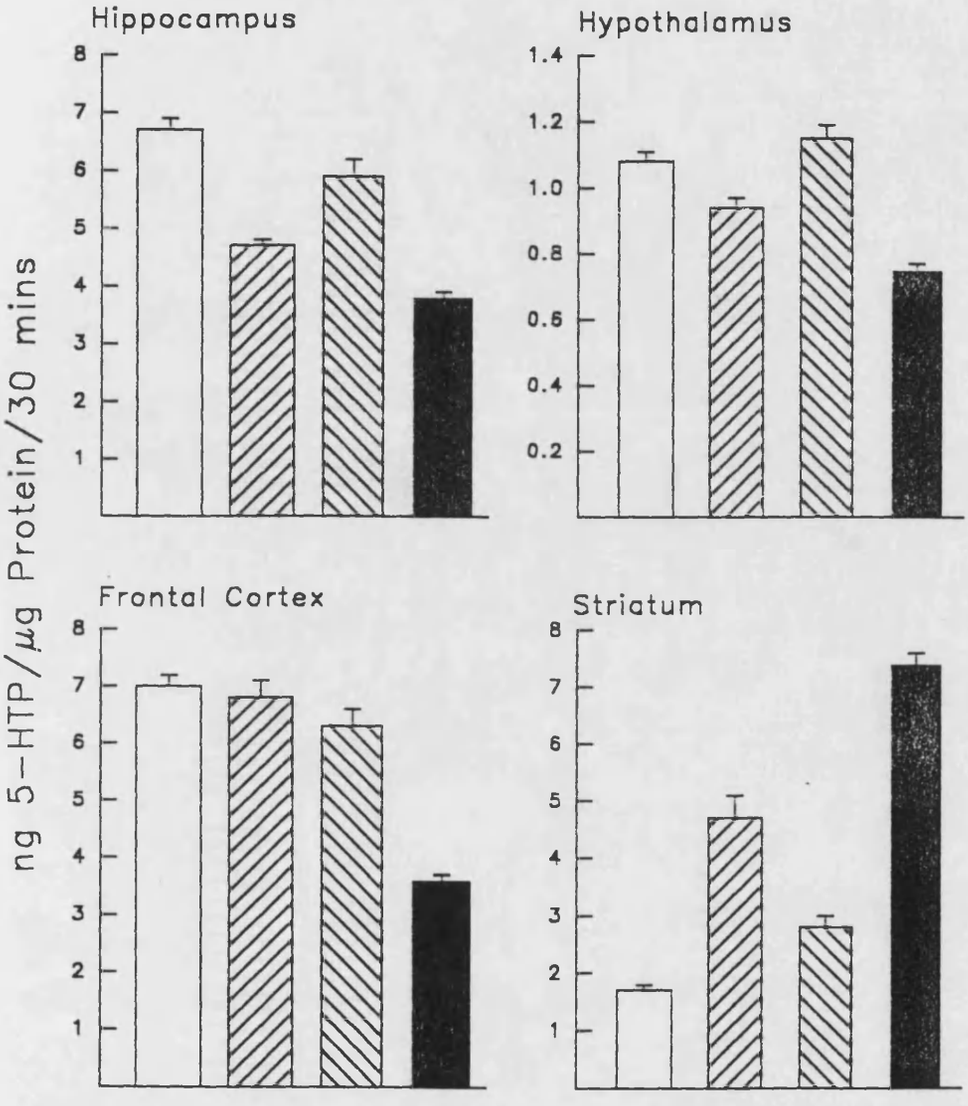


Figure 22. Variation over 24 hours of 5-HTP accumulated after decarboxylase inhibition in the four brain regions studied. Values expressed as ng 5-HTP/µg protein, mean±s.e.m., n=6 for each time point in each brain region, mid light (□), end light (▨), mid dark (▧) and end dark (■). Statistical probabilities of differences in 5-HTP level with time for each brain region are given in table A1 in appendix 1.

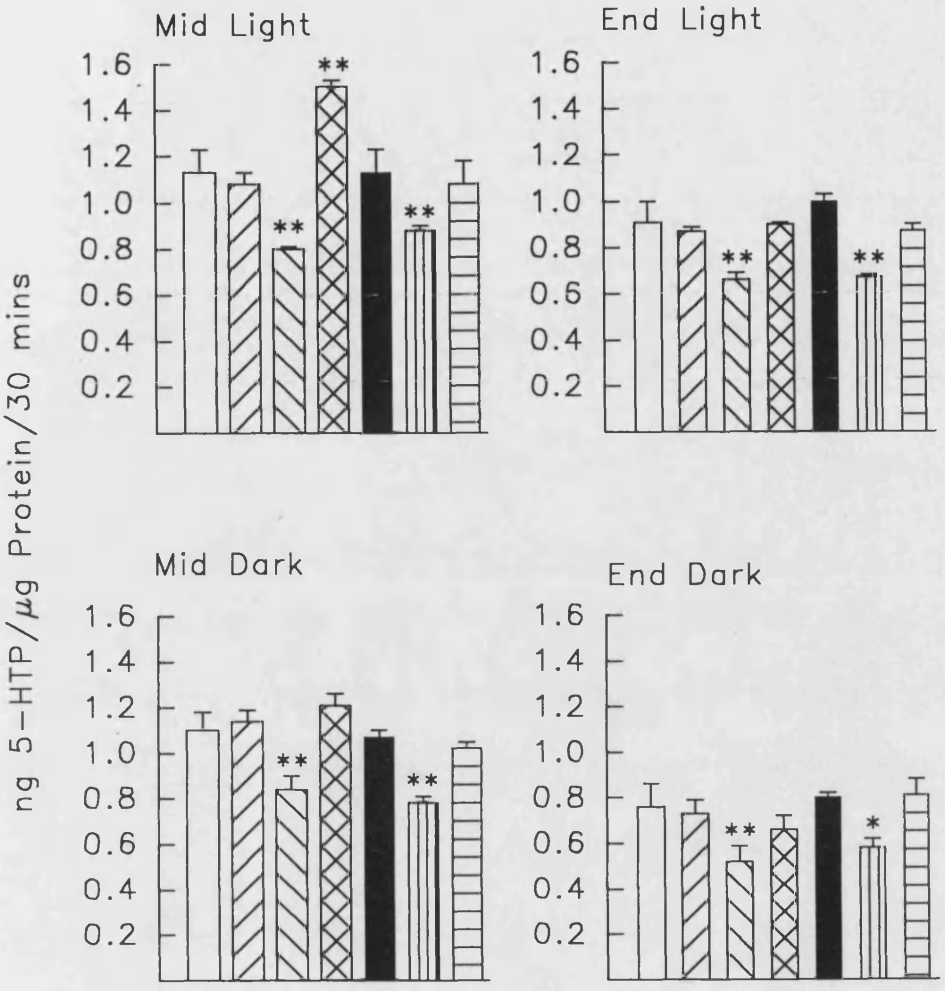


Figure 23. Effect of various pharmacological manipulations on the rate of accumulation of 5-HTP in the **hypothalamus**. Values are expressed in ng 5-HTP/μg protein, as mean±s.e.m., *P<0.05, **P<0.01 vs corresponding vehicle control, n=4-8 depending on time point and treatment, pooled vehicle control (□), 3mg/kg i.p. RU24969 (▨), 9mg/kg i.p. RU24969 (▧), 3mg/kg i.p. (±)cyanopindolol + 9mg/kg i.p. RU24969 (⊠), 3mg/kg i.p. (±)cyanopindolol (■), 5mg/kg s.c. WAY100135 + 9mg/kg i.p. RU24969 (▥), 5mg/kg s.c. WAY100135 (▤).

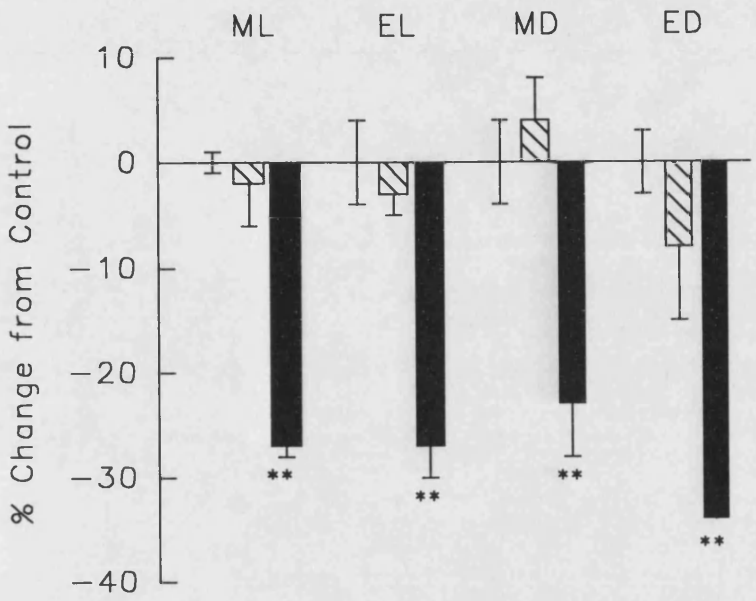


Figure 24. Compound graph of the effects of RU24969 at the four time points in the **hypothalamus.** Values are expressed in % change from corresponding vehicle control value, as mean±s.e.m., *P<0.05, **P<0.01 vs vehicle control, n=4 for each time point and treatment, vehicle control (▭), 3mg/kg i.p. RU24969 (▨) and 9mg/kg i.p. RU24969 (■). ML denotes mid light, EL end light, MD mid dark and ED end dark.

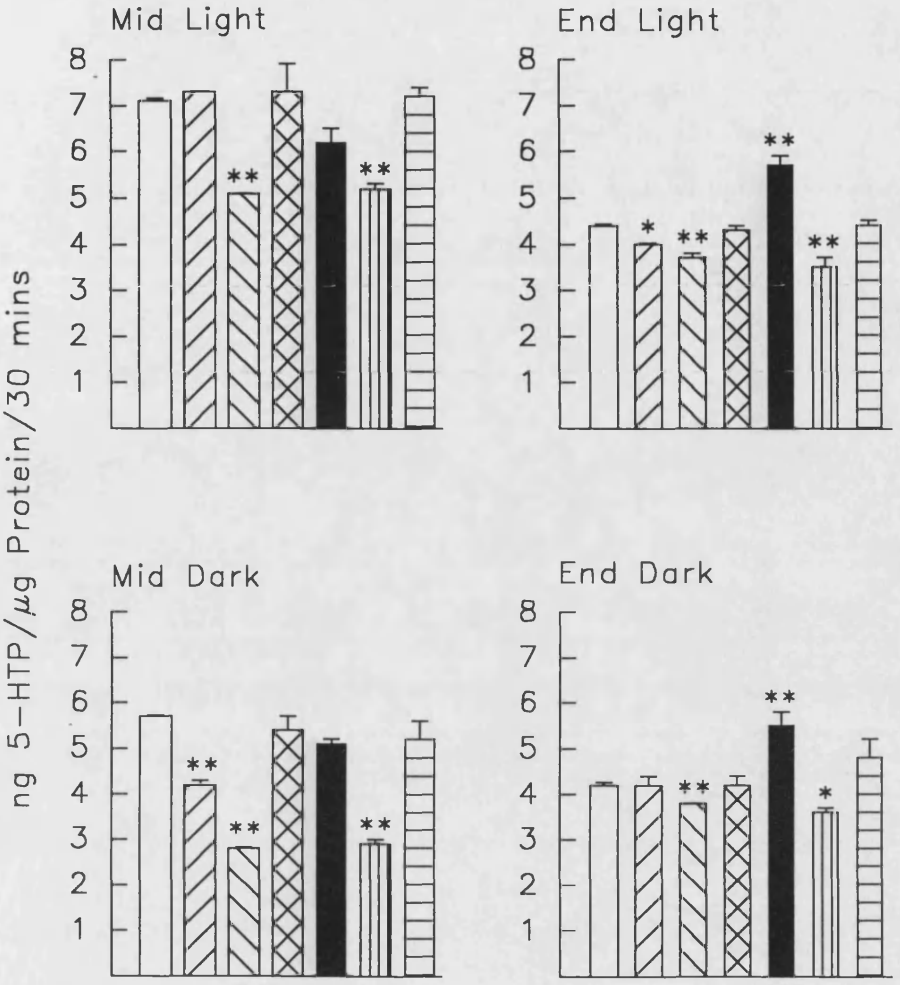


Figure 25. Effect of various pharmacological manipulations on the rate of accumulation of 5-HTP in the **hippocampus.** Values are expressed in ng 5-HTP/µg protein, as mean±s.e.m., *P<0.05, **P<0.01 vs corresponding vehicle control, n=4-8 depending on time point and treatment, pooled vehicle control (□), 3mg/kg i.p. RU24969 (▨), 9mg/kg i.p. RU24969 (▧), 3mg/kg i.p. (±)cyanopindolol + 9mg/kg i.p. RU24969, (▩), 3mg/kg i.p. (±)cyanopindolol (■), 5mg/kg s.c. WAY100135 + 9mg/kg i.p. RU24969 (▥), 5mg/kg s.c. WAY100135 (▤).

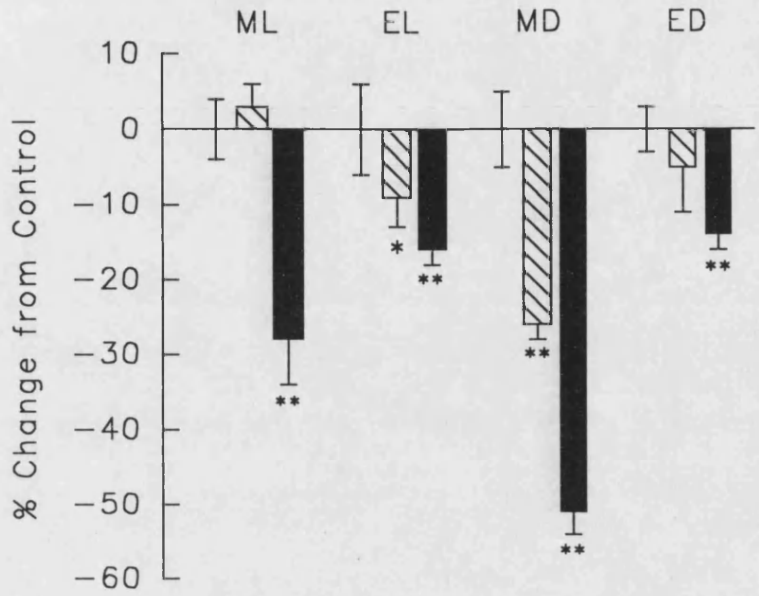


Figure 26. Compound graph of the effects of RU24969 in the **hippocampus.** Values are expressed in % change from corresponding vehicle control value, as mean±s.e.m., *P<0.05, **P<0.01 vs vehicle control, n=4 for each time point and treatment, vehicle control (▭), 3mg/kg i.p. RU24969 (▨) and 9mg/kg i.p. RU24969 (■). ML denotes mid light, EL end light, MD mid dark and ED end dark.

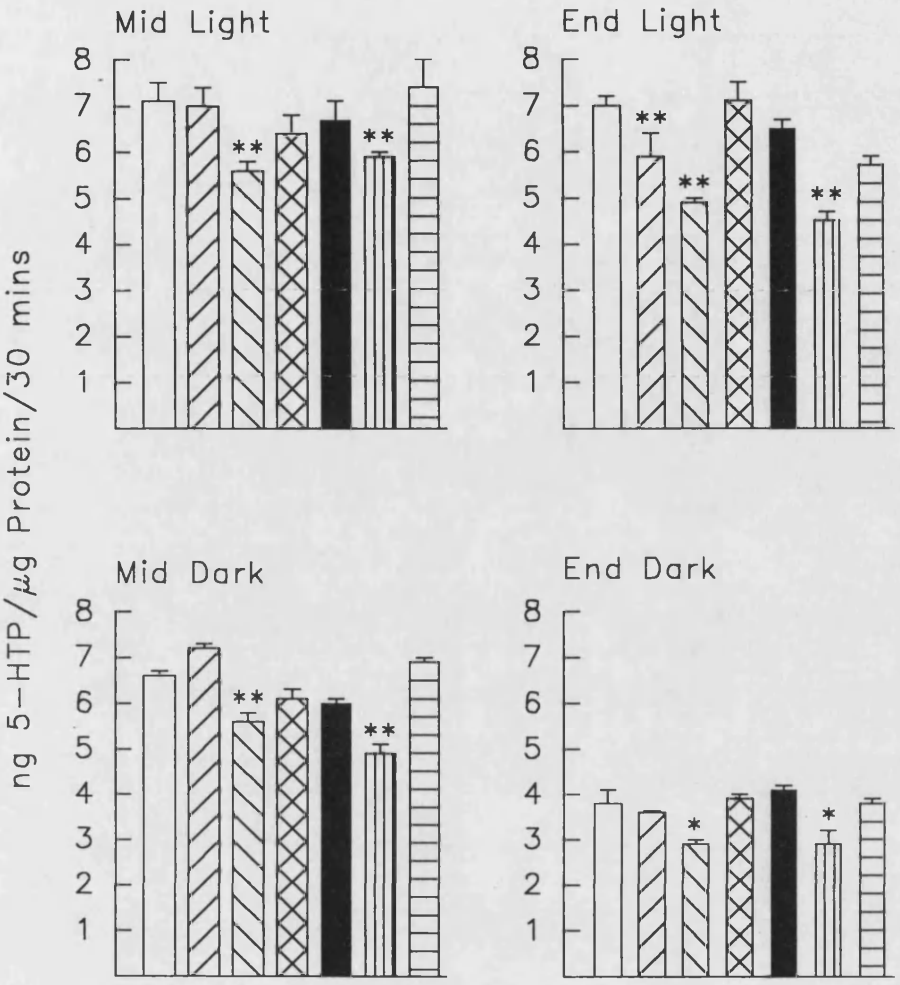


Figure 27. Effect of various pharmacological manipulations on the rate of accumulation of 5-HTP in the **frontal cortex**. Values are expressed in ng 5-HTP/μg protein/30 min, as mean±s.e.m., *P<0.05, **P<0.01 vs corresponding vehicle control, n=4-8 depending on time point and treatment, pooled vehicle control (▭), 3mg/kg i.p. RU24969 (▨), 9mg/kg i.p. RU24969 (), 3mg/kg i.p. (±)cyanopindolol + 9mg/kg i.p. RU24969, (▩), 3mg/kg i.p. (±)cyanopindolol (■), 5mg/kg s.c. WAY100135 + 9mg/kg i.p. RU24969 (▥), 5mg/kg s.c. WAY100135 (▤).

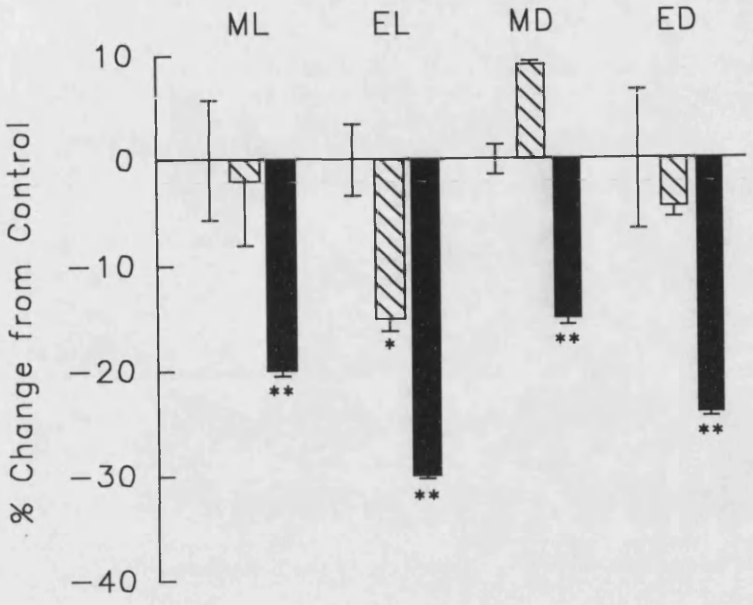


Figure 28. Compound graph of the effects of RU24969 in the **frontal cortex**. Values are expressed in % change from corresponding vehicle control value, as mean±s.e.m., *P<0.05, **P<0.01 vs vehicle control, n=4 for each time point and treatment, vehicle control (□), 3mg/kg i.p. RU24969 (▨) and 9mg/kg i.p. RU24969 (■). ML denotes mid light, EL end light, MD mid dark and ED end dark.

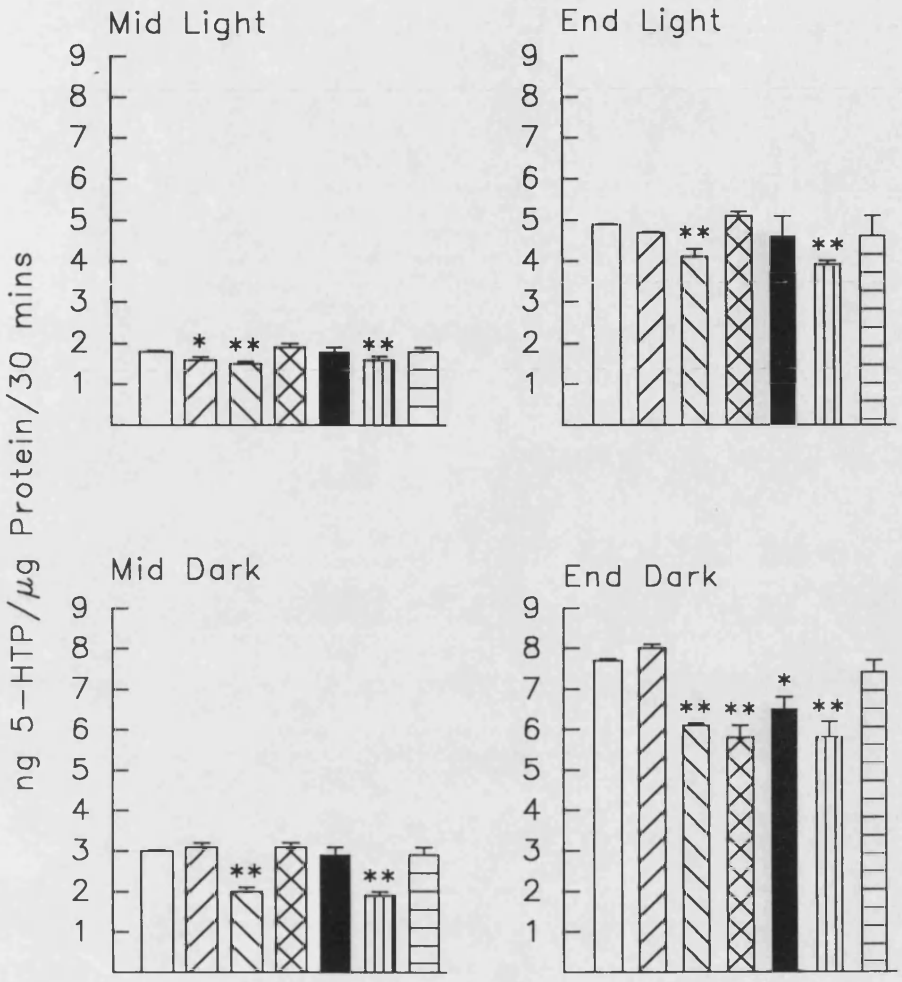


Figure 29. Effect of various pharmacological manipulations on the rate of accumulation of 5-HTP in the **striatum**. Values are expressed in ng 5-HTP/µg protein/30 min, as mean±s.e.m., *P<0.05, **P<0.01 vs corresponding vehicle control, n=4-8 depending on time point and treatment, pooled vehicle control (□), 3mg/kg i.p. RU24969 (▨), 9mg/kg i.p. RU24969 (▧), 3mg/kg i.p. (±)cyanopindolol + 9mg/kg i.p. RU24969 (▩), 3mg/kg i.p. (±)cyanopindolol (■), 5mg/kg s.c. WAY100135 + 9mg/kg i.p. RU24969 (▥), 5mg/kg s.c. WAY100135 (▤).

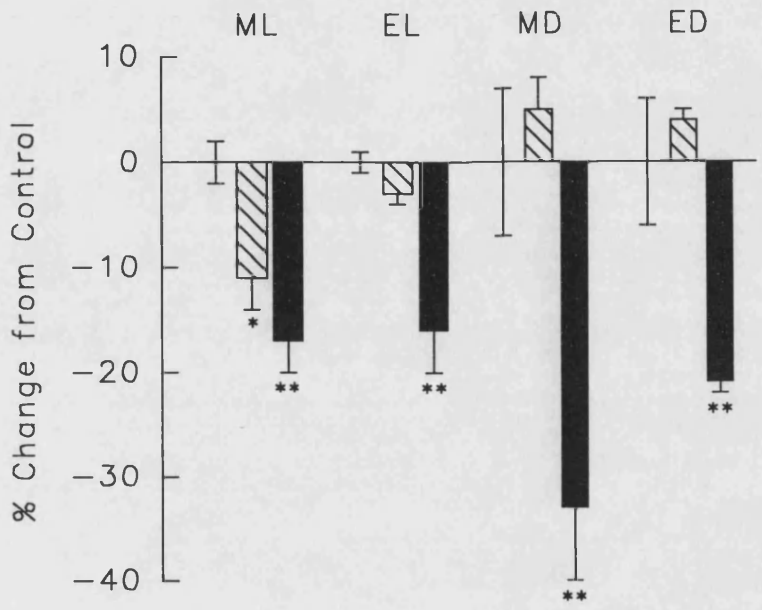


Figure 30. Compound graph of the effects of RU24969 in the **striatum.** Values are expressed in % change from corresponding vehicle control value, as mean±s.e.m., *P<0.05, **P<0.01 vs vehicle control, n=4 for each time point and treatment, vehicle control (▭), 3mg/kg i.p. RU24969 (▨) and 9mg/kg i.p. RU24969 (■). ML denotes mid light, EL end light, MD mid dark and ED end dark.

Chronic antidepressant treatment

Quantitative data tables and details of statistical analysis are presented in Appendix 1.

Basal TrOH activity

Rats were treated chronically with either saline, paroxetine or desipramine to gauge the effect that chronic antidepressant treatment might have on basal TrOH activity. Time and resources only permitted this to be carried out at one time point. End light was chosen because RU24969 (9mg/kg i.p.) was generally most effective in rat brain regions at this time point. Chronic paroxetine or desipramine treatment had no effect on 5-HTPacc compared to saline-treated animals at end light in any of the four brain regions.

Effect of RU24969 in antidepressant-treated animals

Hypothalamus

Chronic paroxetine treatment significantly attenuated the decrease in 5-HTPacc after RU24969 at end light compared with saline-treated animals. Chronic desipramine treatment attenuated the RU24969-induced 5-HTPacc suppression at mid light and end dark compared to saline-treated animals. At the other time points chronic treatment with either antidepressant did not significantly affect RU24969-mediated decrease in 5-HTPacc compared to saline-treated rats, see figure 31 and table A6 of appendix 1.

Hippocampus

Chronic paroxetine treatment significantly enhanced the decrease in 5-HTPacc by RU24969 administration at mid light compared to saline-treated animals. At the other two times chronic paroxetine treatment did not affect the ability of RU24969 to suppress 5-HTPacc. Desipramine did not have any significant affect on 5-HTPacc after RU249696 at any of the four times, compared to saline-treated animals, see figure 32 and table A7 of appendix 1.

Frontal Cortex

The RU24969-mediated decrease in 5-HTPacc was attenuated in paroxetine-treated rats at mid light and mid dark compared to saline-treated rats. At end light, however, prolonged paroxetine treatment enhanced the effect of RU24969. Whilst the RU24969-induced decrease in 5-HTPacc in desipramine-treated animals was enhanced at mid light but attenuated at end light and mid dark compared to saline-treated rats. At the other time points, chronic antidepressant treatment did not significantly affect the RU24969-mediated decrease in 5-HTPacc compared with saline-treated rats, see figure 33 and table A8 of appendix 1.

Striatum

After chronic paroxetine treatment 5-HTPacc following RU24969 administration was enhanced at mid light and attenuated at end light compared to saline-treated animals. Chronic desipramine treatment enhanced the RU24969-induced suppression of 5-HTPacc at end dark but attenuated it at end dark compared to saline-treated rats. Chronic antidepressant treatment had no effect on the RU24969-induced suppression of 5-HTPacc measured at the other time points, see figure 34 and table A9 of appendix 1.

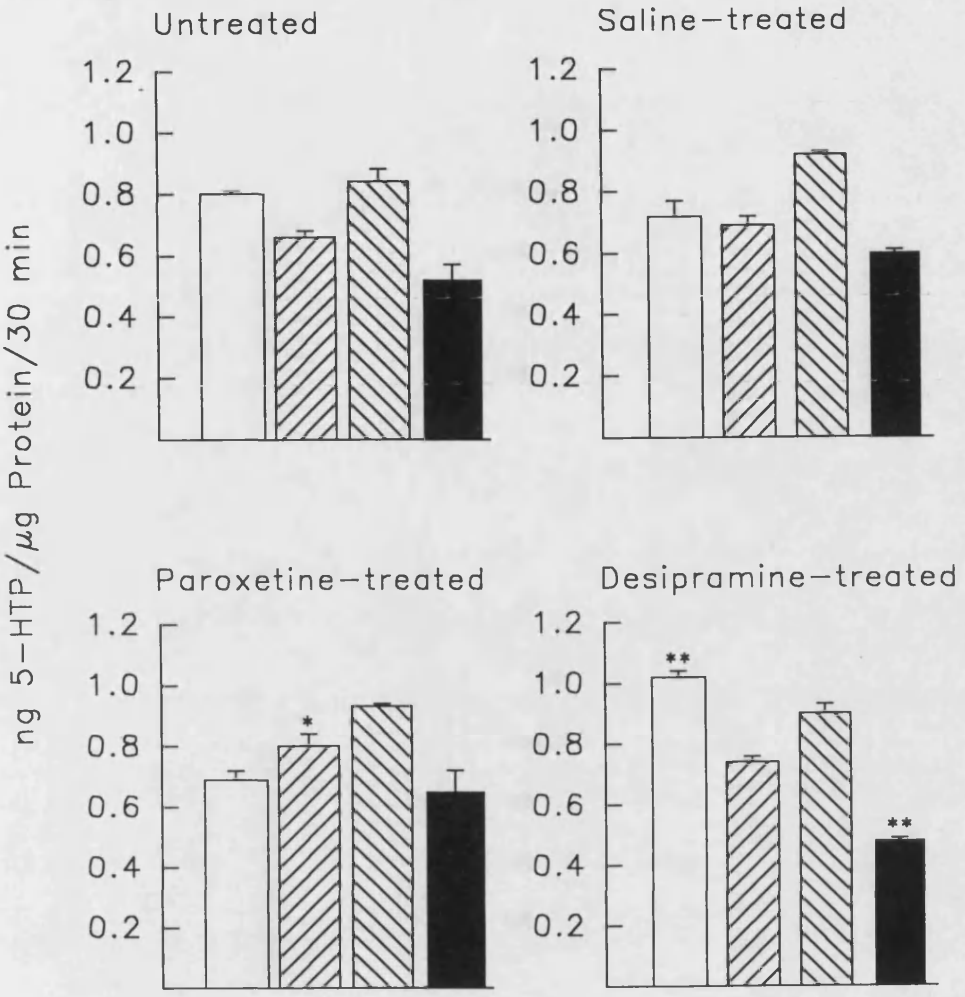


Figure 31. Effect of chronic antidepressant treatment on the pattern of RU24969-induced decrease in the accumulation of 5-HTP in the **hypothalamus** over 24 hours. Values are expressed in ng 5-HTP/μg protein/30 min, as mean±s.e.m., *P<0.05, **P<0.01 vs saline-treated control, n=4 for each time point and treatment, mid light (□), end light (▨), mid dark (▧) and end dark (■). The effect of RU24969 on 5-HTP accumulation over 24 hours in untreated animals, presented in figure 23, has been included for reference.

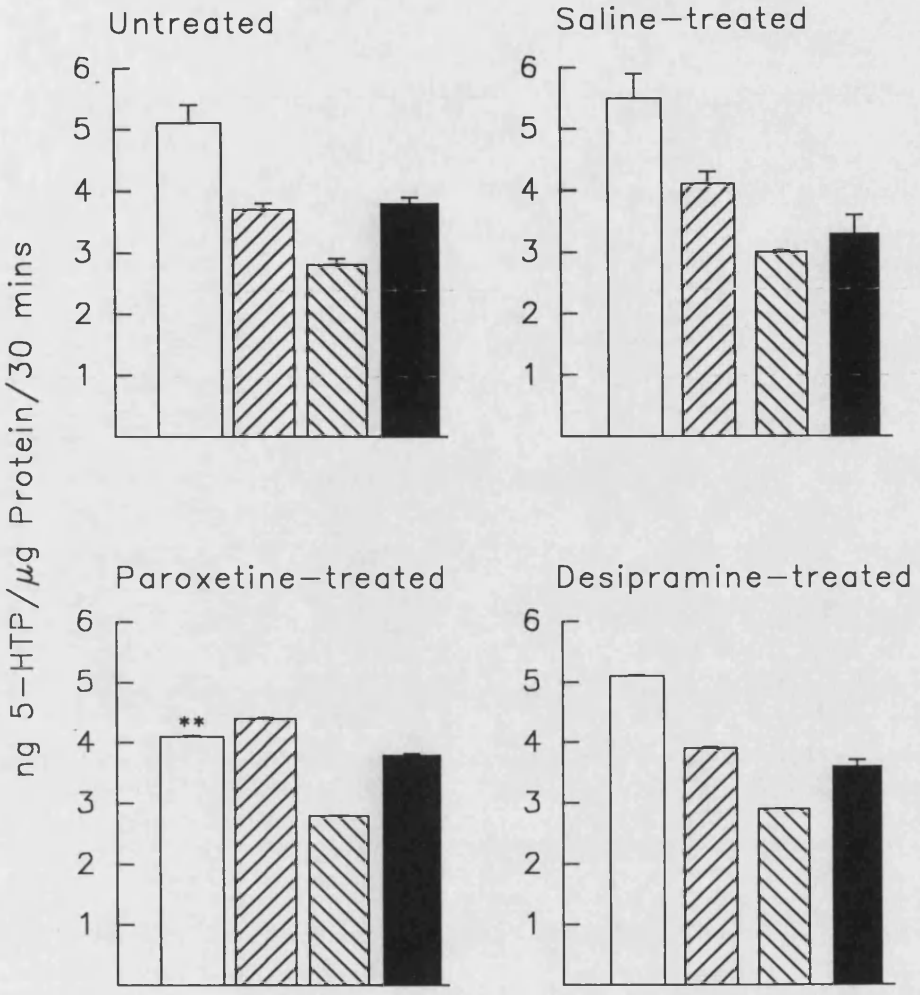


Figure 32. Effect of chronic antidepressant treatment on the RU24969-induced decrease in the accumulation of 5-HTP in the **hippocampus**. Values are expressed in ng 5-HTP/µg protein/30 min, as mean±s.e.m., *P<0.05 vs saline-treated control, n=4 for each time point and treatment, mid light (▭), end light (▨), mid dark (▧) and end dark (■). The effect of RU24969 on 5-HTP accumulation in untreated animals, data repeated from figure 25, is included for reference.

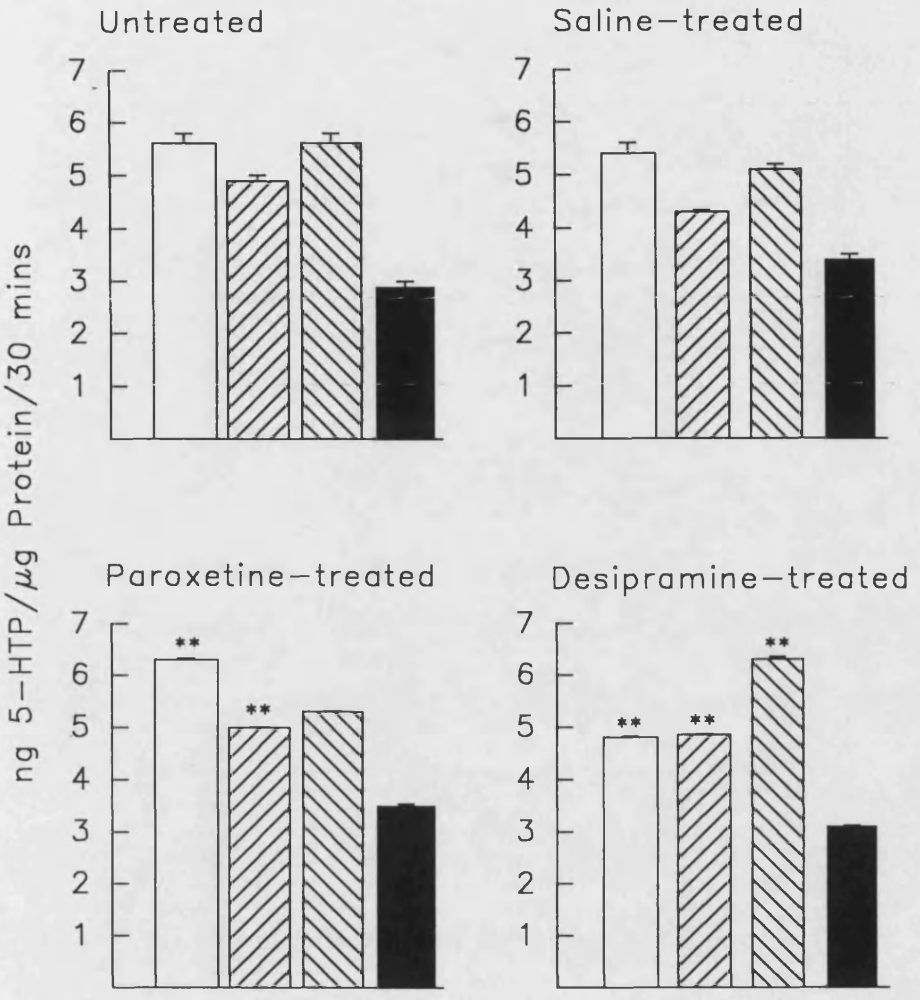


Figure 33. Effect of chronic antidepressant treatment on the RU24969-induced decrease in the accumulation of 5-HTP in the frontal cortex. Values are expressed in ng 5-HTP/μg protein/30 min, as mean±s.e.m., *P<0.05, **P<0.01 vs saline-treated control, n=4 for each time point and treatment, mid light (□), end light (▨), mid dark (▧) and end dark (■). The effect of RU24969 on 5-HTP accumulation in untreated animals, data repeated from figure 27, is given for reference.

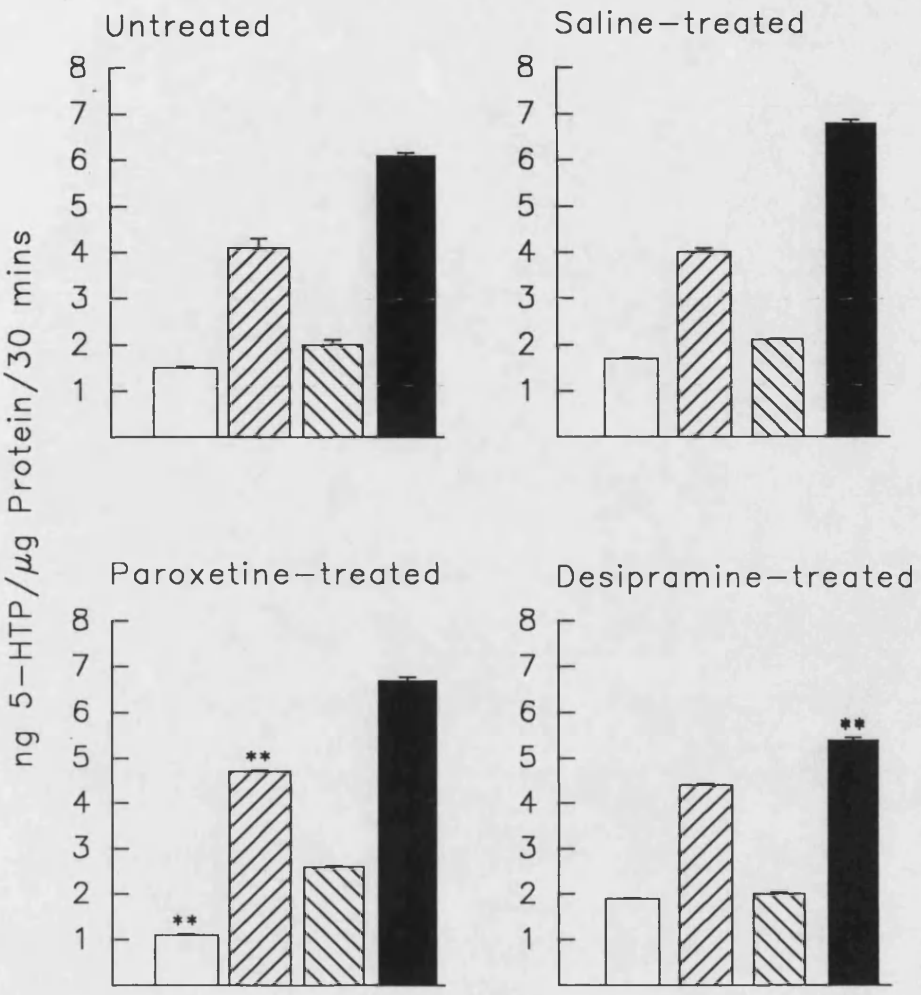


Figure 34. Effect of chronic antidepressant treatment on the RU24969-induced decrease in the accumulation of 5-HTP in the **striatum.** Values are expressed in ng 5-HTP/μg protein/30 min, as mean±s.e.m., *P<0.05, **P<0.01 vs saline-treated control, n=4 for each time point and treatment, mid light (▭), end light (▨), mid dark (▧) and end dark (▬). The effect of RU24969 on 5-HTP accumulation in untreated animals, data repeated from figure 29, is given for reference.

Discussion

The activity of TrOH was gauged over 24 hours by measuring the accumulation of 5-HTP (5-HTPacc) in four brain regions that receive major 5-HT innervation. The regions, hypothalamus, hippocampus, frontal cortex and striatum, were chosen because of this innervation and because 5-HT synthesis has previously been shown to vary significantly over 24 hours in these brain regions (Hery et al. 1972; Kan et al. 1977; Poncet et al. 1993). The results of these experiments demonstrate a variation in both basal synthesis through the light-dark cycle and a 5-HT_{1B}-mediated influence on 5-HT synthesis. Chronic antidepressant treatment affected this 5-HT_{1B}-mediated control of 5-HT synthesis in a phase-dependent manner, as predicted by the hypothesis.

Basal 5-HTP levels

Each of the four brain regions studied here showed a significant variation in basal synthesis rate. There is only one other comparable study measuring 5-HT synthesis rate *in vivo* by the same technique. Poncet and colleagues (1993) demonstrated significant circadian variation in 5-HTPacc in tissue samples obtained by "micropunch" from many brain regions of male OFA rats of comparable weight to the animals used in my experiments. However the brain regions studied do not directly correspond. Poncet and co-workers (1993) found that cortical 5-HTPacc peaked, with a value of 140% of the daily mean, at mid dark having reached a low point, of about 70% of the daily mean, about 3 hours after the end of the light phase. After the peak, 5-HTPacc tailed off from this level to reach a steady level of 100% of the daily mean by the end of the dark phase; this level continuing throughout the light phase. However, the data presented here for the frontal cortex show a different pattern of 5-HTPacc; equally high levels were measured throughout the light phase (106-118% of the daily mean) with a trough at the end of the light phase, 61% of the daily mean. Poncet and colleagues measured 5-HTPacc in two hypothalamic nuclei; the suprachiasmatic (SCN) and the ventromedial (VMN). In both regions, 5-HTPacc peaked at mid dark, with a value of 140% of the daily mean, and reached a trough at

mid light (70% and 80% of the daily mean for the SCN and VMN respectively). In the basal hypothalamus, however, a different pattern was observed; peak accumulation occurred at mid dark (117% of the daily mean) while the lowest 5-HTPacc was measured at end dark (76% of the daily mean). The differences may be accounted for by the differences in brain region, e.g. cortex vs frontal cortex, or by differences in sampling frequency (Poncet and colleagues sampled every 4 hours whilst I sampled every 6 hours) or by the method of obtaining samples, dissection vs micropunches.

The only other published studies of 5-HT synthesis used homogenates or sonicates derived from rats killed at the appropriate phase of their light-dark cycle. However the photoperiod, strain and methodologies used vary significantly.

Significantly higher 5-HT synthetic rates in the light phase have been reported in the hypothalamus and cortex *in vivo* and in slices *in vitro* (Hery et al. 1972). However it would appear that the observed rhythm was probably a result of their methodology which measured [^{3}H]5-HT and [^{3}H]5-HIAA levels after administration of [^{3}H]tryptophan, the uptake of which has itself been shown to vary over 24 hours (Hery et al. 1972; Liozou and Redfern 1986). The conversion index of 5-HT was the same during the light phase and dark phase (Hery et al. 1972).

Using striatal sonicates from OFA rats of comparable weight, Kan and co-workers (1977) demonstrated a significant circadian variation in TrOH activity *in vitro*. The pattern observed was dissimilar from the data presented in this thesis; peak activity was measured at mid light (150% of daily mean) and was lowest at mid dark (50% of the daily mean). McLennan and Lees (1978), however, were unable to detect a rhythm in TrOH activity in striatal homogenates from male Sprague-Dawley rats using a different method from Kan and colleagues (1977).

The regional differences in 5-HT synthesis rate through the light-dark cycle may reflect the role of 5-HT in those brain regions. However there is not very much research in this area of 5-HT.

The firing rate of 5-HT neurons in the RN has been considered to be correlated to the behavioural state of that animal (Trulson and Jacobs 1979). In rats (Imeri et al.

1994) and cats (Trulson and Jacobs 1979; Wilkinson et al. 1991) neuronal firing rate increases with increasing levels of consciousness. However the terminal release of 5-HT has been found to be both related to (Imeri et al. 1994) and independent of (Kalen et al. 1989) the activity state. There is some evidence that 5-HT synthesis might be related to neuronal activity. This theory stems from two observations; firstly electrical stimulation of the DR or MR increases TrOH activity *in vivo* (Petersen et al. 1989) and secondly administration of 5-HT_{1A} agonists, which decrease neuronal firing, also decrease 5-HT synthesis (Invernizzi et al. 1991). Interestingly the increase in 5-HT synthesis induced by l-try administration, as outlined on page 12 of the introduction, was blocked by concurrent administration of 8-OH-DPAT (Fernstrom et al. 1990), also implying that neuronal activity is required for synthesis, and a mechanism involving cAMP cannot be ruled out, see later.

The results presented here are not in agreement with the theory that 5-HT synthesis parallels neuronal activity. Generally synthesis rates were found to be higher during the light phase a time when RN firing rates are low, but each region showed its own characteristic pattern of 5-HTPacc. The synthesis rate could be affected by three differing factors.

First, handling and injection of the rats and the stress invoked may affect 5-HT release/synthesis. Rats were handled every 2-3 days whilst they were phase-shifted in order to habituate them and to reduce stress on human contact. However, rats handled during their light phase would be expected to be stimulated more than rats handled during their dark phase. The resultant stimulation might increase neuronal firing rate. However it has been shown in cats that restraint and introducing a dog into their vicinity has no effect on dorsal raphe neuronal firing (Jacobs and Azmitia 1992), even though there is a normal behavioural response. On the other hand handling rats has been shown both to increase 5-HT release in the hippocampus (Kalen et al. 1989) and to have no effect on extracellular 5-HT levels in the frontal cortex and DRN (Clement et al. 1993). Any stress-induced 5-HT release might in turn cause feedback to inhibit 5-HT synthesis; the effect of this feedback could vary in different brain regions.

The brain regions studied here receive innervation from both RN, with the exception of the striatum which receives only a DRN input. It is well known that the fibres from the DR and MR are neuroanatomically distinct, see page 3 of the introduction. Additionally the RN possess a high degree of topographical organisation (Jacobs et al. 1978; van Bockstaele et al. 1993). For example the DR input to the amygdala and hippocampus stems from the caudal wings of the DRN (Imai et al. 1986). The behavioural state/neuronal firing relationship, as outlined earlier, has been demonstrated in both MR and DR, however not all 5-HT neurons display this relationship. There are subsets of DR and MR neurons that are not clocklike i.e. do not show the relationship between state of arousal and firing rate, which are scattered throughout the nuclei (Shima et al. 1986). It is therefore conceivable that the regional pattern of 5-HTPacc reflects preferential innervation from one RN rather than another and therefore the type of 5-HT fibre and the number of non-clocklike neurons projecting to that area might contribute to the effect of topographical innervation.

The RN receive many afferents which can influence the neuronal firing rate, for example noradrenaline (NA) is thought to have a tonic excitatory effect on 5-HT neuronal firing rate mediated by a_1-adrenoceptors. Interestingly, in the DR some of these afferents only exert an effect during certain behavioural states. The GABA input mediates suppression of neuronal activity only during slow wave sleep, the NA input is maximal during wakefulness whilst the EAA input only affects firing rate in response to auditory stimulus (Levine and Jacobs 1992). It is conceivable that these inputs might affect neuronal firing differently over the sleep-wake cycle and thus affect 5-HT synthesis. In addition some afferent-mediated control may be evoked during handling and injection.

It is unlikely that tryptophan availability accounts for changes in basal 5-HTPacc in the four brain regions studied. It has previously been shown that if food is restricted to a few hours during the light phase then the circadian rhythm in brain 5-HT content is not significantly different from animals allowed free access to food (Morgan and Yndo 1973). In addition, although plasma l-try varies over 24 hours, the kinetics of

the neuronal l-try transporter also vary and in effect buffer the entry of l-try into the neuron (Loizou and Redfern 1986).

Agonist and Antagonist Studies

Pharmacology

There is a paucity of selective 5-HT_{1B} ligands; RU24969 was chosen since it was the agonist with least affinity for other receptors (see van Wijngaarten et al. 1990).

At a dose of 3mg/kg RU24969 significantly reduced 5-HTPacc in some brain regions at some time points, whilst RU24969 (9mg/kg i.p.) significantly reduced the rate of formation of 5-HTP in all brain regions at all time points. Although the latter dose is high (Euvrard and Boissier 1980) it was used because 3mg/kg i.p. RU24969 had little effect in the hypothalamus. Animals that received the higher dose showed marked 5-HT syndrome with flat body posture, hyperlocomotion, Straub tail and sweating. RU24969 (1-10mg/kg) has previously been shown to cause a dose-dependent decrease in 5-HTPacc after decarboxylase inhibition (Euvrard and Boissier 1980). At the maximal dose, accumulation was decreased by 34% and 35% in the brainstem and forebrain respectively. In the experiments performed in this thesis maximal inhibition of 5-HTPacc by RU24969 (9mg/kg) was 44% in the hypothalamus, 51% in the hippocampus, 30% in the frontal cortex and 33% in the striatum.

RU24969 has only slightly greater affinity for the 5-HT_{1B} receptor than the 5-HT_{1A} autoreceptor (pK_i 5-HT_{1A} 8.06nM and pK_i 5-HT_{1B} 8.23nM van Winjgaarten et al. 1990). Pre-administration of (±)cyanopindolol, a 5-$HT_{1A/1B}$ antagonist (Schlicker et al. 1985 pA_2 8.3 at the terminal autoreceptor; Neill and Cooper 1989), blocked the effects of RU24969, but the RU24969-induced suppression of 5-HTPacc may still have been due to effects on both the 5-HT_{1A} and 5-HT_{1B} receptors. Therefore further receptor characterisation was performed using (+)WAY100135, a novel silent 5-HT_{1A} antagonist (Fletcher et al. 1993; Routledge et al. 1993; Lanfumey et al. 1993). (+)WAY100135 was given either alone or in conjunction with RU24969 to gauge what proportion of the decrease in 5-HTPacc after administration of RU249696 might be

attributable to stimulation of 5-HT_{1A} receptors. (+)WAY100135 did not block the 5-HTPacc-suppressing effects of RU24949 (9mg/kg i.p.) and (+)WAY100135 alone had no effect on 5-HTPacc, as previously reported (Fletcher et al. 1991). This indicates that stimulation of a 5-HT_{1A} receptor had very little contribution to the effect of RU24959 and that its function did not apparently vary with time of day. Therefore it can be concluded that the RU24969-induced decrease in 5-HTPacc is mediated by a 5-HT_{1B} receptor.

To summarise, the 5-HT receptor affecting 5-HTPacc was pharmacologically characterised as being a 5-HT_{1B} receptor since the effects of RU24969, a 5-$HT_{1A/1B}$ agonist, were blocked by the 5-$HT_{1A/1B}$ antagonist (±)cyanopindolol but not the selective 5-HT1A anatgonist WAY100135.

One factor that must not be overlooked is the effect of 5-HT_{1B} ligands on dopamine or noradrenaline synthesis and release, since the decarboxylase inhibitor used blocks the conversion of DOPA to dopamine. Accumulated DOPA might leave dopaminergic and noradrenergic neurons and interfere with TrOH activity, although this effect is known to be minimal (Nissbrandt et al. 1988). Experimental evidence indicates that 5-HT_{1B} ligands have no significant effect on the firing rate of nigrostriatal dopaminergic neurons (Kelland et al. 1990) and 5-HT_{1A} ligands do not affect the firing rate of substantia nigra neurons (Nissbrandt et al. 1988). However RU24969 decreases noradrenergic neuronal firing (Clement et al. 1992a) which might affect the tonic excitation of α_1-heteroreceptors. There is one report of a 5-HT_{1B} agonist increasing DOPA accumulation after AAAD inhibition (Hjorth 1993). However the 5-HT_{1B} agonist used shows less discrimination between 5-HT_{1A} and 5-HT_{1B} receptors than does RU24969 (van Winjgaarten et al. 1990).

5-HT heteroreceptors have been demonstrated to affect ACh (Maura and Raiteri 1986), DA (Ennis et al. 1981) and NA terminals (Feuerstein and Hertting 1986). 5-HT_{1B} receptors decrease ACh release whilst a 5-HT_3 receptor mediates the effects of 5-HT on DA and NA release *in vitro* (Blandina et al. 1989). One *in vivo* microdialysis study has reported that RU24969, infused via the probe, increases

dialysate DA levels (Benloucif and Galloway 1991). However at the concentration of RU24969 used (50μM), RU24969 would affect all subtypes of the 5-HT_1 receptor, 5-HT_2 and 5-HT_3 receptors, as well as α- and β-adrenoceptors, and D_2 receptors.

The location of the 5-HT_{1B} receptor responsible for the suppression of 5-HT synthesis was not directly determined. There are two possible locations, either presynaptically or post-synaptically on a neuron which in turn impinges on and influences the 5-HT neuron. The two possibilities are shown in figure 35; the putative mechanism for presynaptic inhibition of inhibition of TrOH is described later. In the indirect mechanism 5-HT_{1B} stimulation inhibits the release of a neurotransmitter that tonically activates serotoninergic neurons. The inhibition of this tonic excitatory effect could in turn causes a decrease in the activity of TrOH. One candidate could be an ion channel. Nicotinic acetylcholine receptors are found presynaptically on 5-HT terminals; however nicotinic antagonists have no effect on [^{3}H]5-HT release (Hery et al. 1977a). A $GABA_A$ receptor is also ruled out since $GABA_A$ agonists and antagonists do not affect [^{3}H]5-HT release (Schlicker et al. 1984). Other possible candidates are receptors linked to the stimulation of cAMP, e.g. β-adrenoceptors or D_1 dopamine receptors. There is no evidence of presynaptic β-adrenergic or dopaminergic heteroreceptors on affecting 5-HT terminal release (Frankhuyzen and Mulder 1980; Gross et al. 1987). A further alternative would involve a post-synaptic 5-HT_{1B} receptor located on the cell body of a neuron which synapses on a second neuron that itself impinged onto the 5-HT neuron. The neurotransmitter of this second neuron could be either excitatory or inhibitory depending on the nature of the interneuron. On balance the presynaptic autoreceptor model is more likely since autoreceptors modulating release have been demonstrated on synaptosomes from the hypothalamus (Martin and Sanders-Bush 1982). To define the location of the receptors, 5-HTPacc in synaptosomes could be measured to indicate whether the receptor responsible was located presynaptically. Alternatively antisense deoxyoligonucleotides for the 5-HT_{1B} receptor could be injected into the RN to remove presynaptic 5-HT autoreceptors.

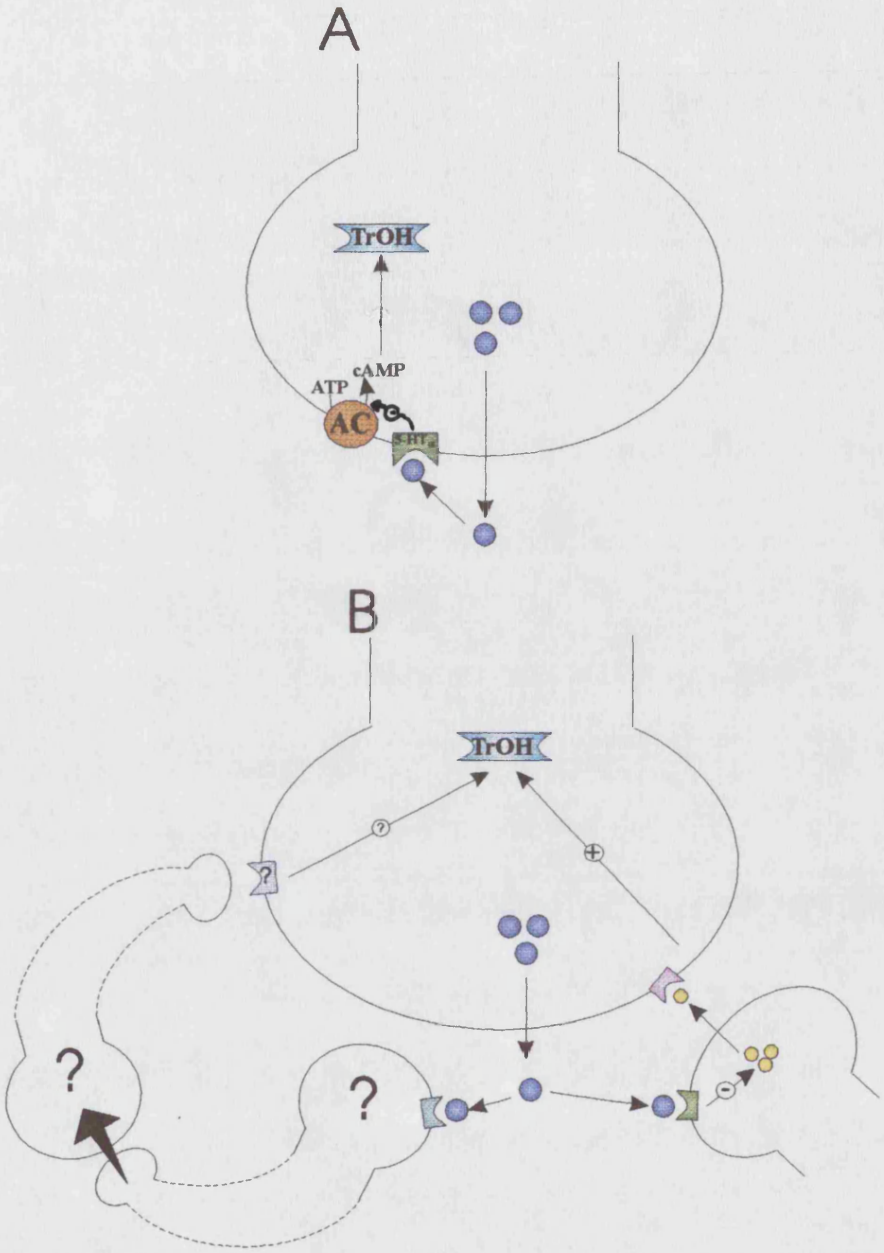


Figure 35. Two models to explain how the RU24969-mediated decrease in 5-HTP accumulation might be achieved. **A**. The effect of RU24969 is mediated by a presynaptic 5-HT_{1B} autoreceptor, negatively linked to cAMP **B**. The 5-HT_{1B} receptor is located post-synaptically on a another nerve terminal that impinges on the 5-HT terminal. The neurotransmitter released from this post synaptic neuron would normally exert a tonic excitatory effect on 5-HT neurons. Stimulation of the inhibitory 5-HT_{1B} heteroreceptor decreases the release of the excitatory neurotransmitter thus removing the tonic stimulation. Alternatively the 5-HT_{1B} heteroreceptor might be located on the cell body of an interneuron which in turn affects the release of second interneuron which then affects the 5-HT neuron.

Proposed mechanism of the 5-HT_{1B}-induced decrease in TrOH

Hamon and colleagues (1973) produced the first evidence that 5-HT might be controlling its own synthesis. Measuring the rate of [^{3}H]5-HT synthesis from [^{3}H]l-try in striatal slices, they found that exogenously applied 5-HT could reduce the production of [^{3}H]5-HT. The effect was not inhibited if a selective 5-HT uptake inhibitor was included in the buffer, indicating that the effect was mediated extracellularly. It has recently been demonstrated that this effect is mediated by a 5-HT_{1B} (auto)receptor (Hjorth 1993) and was resistant to hemitransection and reserpine pre-treatment. These results demonstrate that the effect was not dependent on nerve impulses and was directly mediated by a receptor.

The mechanisms controlling TrOH activity have not been investigated in any detail and are poorly understood. TrOH is phosphorylated by cAMP-dependent protein kinase (PKA) and calcium/calmodulin-dependent protein kinase (Ca^{++}/CaM PKII) in the presence of an activator protein, protein 14-3-3 (Yamauchi et al. 1981; Makita et al. 1990). Protein 14-3-3 alone has no effect on PKA or Ca^{++}/CaM PKII-dependent phosphorylation of TrOH; but protein 14-3-3 binding appears to be responsible for protein kinase-dependent activation (Furukawa et al. 1993). If it is accepted that the effect of RU24969 is mediated by a 5-HT_{1B} autoreceptor, the results suggest that the control is exerted at the level of TrOH since the decarboxylation step was inhibited. A possible mechanism is shown in figure 36. The activity of at least one type of adenylate cyclase (AC; see later) is known to be tonically stimulated by calcium/calmodulin. The activity of this enzyme would be inhibited by the released α_i, and perhaps $\beta\gamma$ subunits of the G_i-protein which dissociate when the 5-HT_{1B} receptor is stimulated, as described on page 10 of the introduction. A decrease in the activity of adenylate cyclase would decrease the intracellular concentration of cAMP and therefore PKA activity. It has also been proposed that the 5-HT_{1B} autoreceptor decreases the intracellular influx of Ca^{++} through voltage-dependent calcium channels

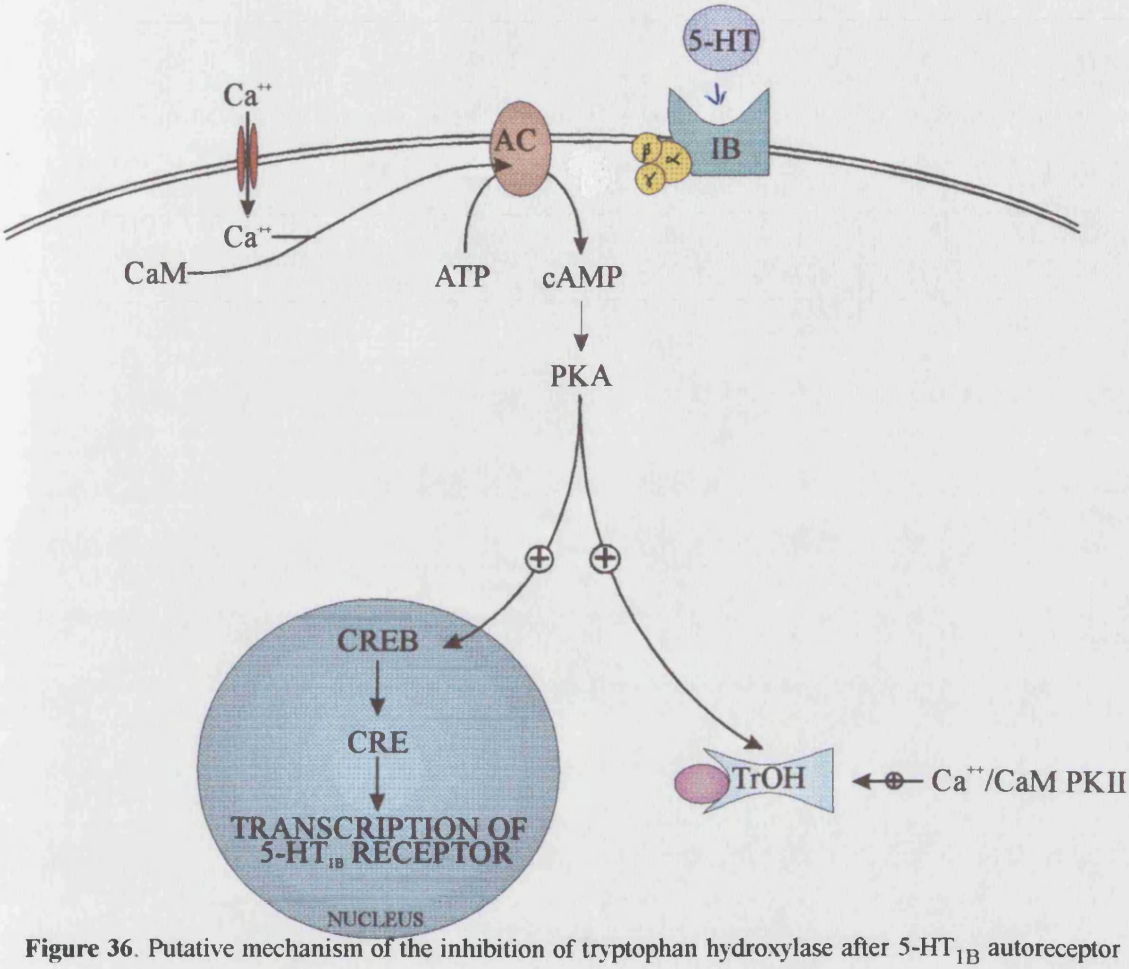


Figure 36. Putative mechanism of the inhibition of tryptophan hydroxylase after 5-HT_{1B} autoreceptor stimulation. When 5-HT binds to its G protein-linked autoreceptor (yellow trilobed structure), it causes the dissociation of the G_i into its α, β and γ subunits which inhibit adenylate cyclase (AC, orange oval) activity. The inhibition of this basally active enzyme decreases the intracellular level of cAMP and therefore PKA stimulation. Under normal conditions, therefore, there is a basal stimulation of tryptophan hydroxylase (TrOH, turquoise shape) by PKA which is prevented after 5-HT_{1B} autoreceptor stimulation. Additionally 5-HT_{1B} mediated closure of calcium channels (red oblong) might prevent the basal activation of TrOH by calcium/calmodulin-dependent protein kinase II (Ca^{++}/CaM PKII). Activation of TrOH requires the presence of an activator protein (pink sphere). PKA is also thought to regulate the rate of transcription of certain genes, perhaps including the 5-HT_{1B} receptor, via a cAMP response element-binding protein (CREB) at the cAMP response element (CRE) located in the regulatory domain of certain genes, this aspect is more fully explained in the text.

Regional Differences

There are several possible explanations for the regional differences in the effect of RU24969.

The simplest explanation is that the concentration of RU24969 in some brain regions was higher than in others because of differential blood supply and cell density. This could be ascertained by measuring RU24969 levels in the specific brain areas after peripheral administration using either microdialysis or homogenates and HPLC.

The differences may be attributable to a regional variation in the distribution and density of terminal 5-HT_{1B} autoreceptors. There are no reports on the regional distribution of presynaptic 5-HT_{1B} receptors, which makes interpretation of the results more speculative. However the total number of 5-HT_{1B} binding sites has been measured and, in decreasing B_{max}, the rank order of distribution is hypothalamus>caudate-putamen>frontal cortex>hippocampus (Bruinvels et al. 1993). These findings do not correlate well with the regional effects of RU24969 in my experiments. For example the hypothalamus an area rich in 5-HT_{1B} receptors showed less sensitivity to RU24969 than the frontal cortex, whereas the hippocampus, with the lowest total 5-HT_{1B} binding, proved to be the area most affected by RU24969. Alternatively the K_D of 5-HT autoreceptors might vary between regions, opening up the possibility that 5-HT synthesis is more tightly controlled in some regions than others. The results indicate that over the light-dark cycle the hippocampus shows the greatest degree of control, closely followed by the frontal cortex, whilst the hypothalamus shows the least. Superfusion studies employed by Middlemiss (1984) in frontal cortex, striatum and hippocampus showed that the degree of inhibition of [^{3}H]5-HT release after addition of 1μM 5-HT varied between region. Exogenous 5-HT added to frontal cortical slices inhibited release by 63%, in the hippocampus by 58% and in the striatum by 46%. If my results at mid light are compared to those of Middlemiss (1984) then the striatum shows least inhibition and the hippocampus most, although the frontal cortex value was close (hippocampus 28%, hypothalamus 27%, frontal cortex 21% and striatum 17%). The number and affinity of binding sites

throughout the brain could be measured by binding studies using 5-HT, RU24969 or cyanopindolol as ligands, or quantitative *in situ* hybridisation.

Secondly the differential regional response to RU24969 may be due to effects on the somatodendritic 5-HT_{1A} and 5-HT_{1B} receptors in the DRN and MRN. 5-HT_{1B} receptors are found in the raphe nuclei (Neumaier et al. 1993) so peripherally administered RU24969 is free to act at these sites. As explained earlier RU24969 is not selective for 5-HT_{1B} receptors. Since RU24969 was administered peripherally the effects of RU24969 may in part be due to stimulation of the raphe somatodendritic 5-HT_{1A} and 5-HT_{1B} autoreceptors. However the results from the study using (+)WAY100135 indicate that most of the RU24969-induced decrease in 5-HTPacc is due to stimulation of 5-HT_{1B} receptors.

Somatodendritic 5-HT_{1A} autoreceptors can affect synthesis and terminal release of 5-HT (Invernizzi et al. 1991). Some experiments have suggested that the sensitivity of the somatodendritic 5-HT_{1A} receptor differs in the DRN and MRN (Sinton and Fallon 1988; Invernizzi et al. 1991). In areas that receive innervation from both the DRN and MRN, such as the hippocampus and hypothalamus, stimulation of the 5-HT_{1A} receptors in the DRN had no effect on 5-HTPacc, whereas stimulation of the MRN decreased 5-HTPacc (Invernizzi et al. 1991). There are some early indications that the 5-HT_{1A} receptor subdivision might comprise two receptors (Radja et al. 1992; Blier et al. 1993a, b). Radja and colleagues propose that the two types of 5-HT_{1A} receptor occur both presynaptically and post-synaptically. Perhaps one type of 5-HT_{1A} receptor is more prevalent in one area of the RN than the other, and, given the topographical organisation of the RN, provides more autoreceptor control for one brain area than the other type of 5-HT_{1A} receptor. Interestingly, peripheral administration of 5-HT_{1B} agonists can also influence the firing rate of DRN and MRN neurons differentially (Sinton and Fallon 1988). In the DRN the agonists decreased the firing rate, but in the MRN the agonists had biphasic effects, low concentrations caused excitation, whilst higher concentrations had either inhibitory effects or returned firing rates back to baseline. Measurement of the effect of microinjection of RU24969 in the

DR or MR on 5-HTPacc in various brain region could be used to determine the contribution of somatodendritically located 5-HT_{1B} receptors in the RU24969-induced decrease in 5-HTPacc.

Since RU24969 was administered peripherally, it would have to compete with endogenous 5-HT for binding to 5-HT_{1B} receptors. Therefore the contribution of endogenous 5-HT to the observed effect would depend on its biophase concentration. More 5-HT might be released in some brain regions than others, although it might be expected that autoreceptor number or sensitivity might also vary, in effect cancelling out this effect. The level of 5-HT in each brain region and the sensitivity of the autoreceptor in that region could be determined by *in vivo* microdialysis.

Regional differences could be due to differences in the adenylate cyclase type coupled to the 5-HT_{1B} receptor. To date, there are at least 6 distinct types of adenylate cyclase (AC) which show distinct regional localisations and sensitivities to Ca^{++}, CaM and G-protein coupled receptors (Choi et al. 1993; Pieroni et al. 1993).

Type I AC (AC I) is located in the hippocampus CA2 region and to a lesser extent in the neocortex. AC I is the best studied and characterised AC isozyme. It is basally stimulated by Ca^{++}/CaM and inhibited by free $\beta\gamma$ G protein subunits. Type II AC (AC II) is found in the hippocampus regions CA1-4 and the hypothalamus and is the most unusual type. It is inhibited by $G_i\alpha$ and this inhibition can be overcome by protein kinase C-induced stimulation; additionally free $\beta\gamma$ subunits released after G_i dissociation stimulate AC II. Type III AC is found in low levels throughout the hippocampus; it is basally stimulated by CaM and is insensitive to bg subunits. Type V AC (AC V) is only found in the striatum and is inhibited by low intracellular Ca^{++} levels and $G_i\alpha$, it is insensitive to free $\beta\gamma$ subunits. It has been postulated that AC V is inhibited by PKA (Choi et al. 1993; Pieroni et al. 1993). The presence and relative amounts of the different types of AC would therefore determine what effect 5-HT_{1B} receptor stimulation would have on cAMP levels intracellularly. If the amount of each type of AC could be measured after selective destruction of the 5-HT system by 5,7-dihydroxytryptamine, it might help to define the AC linked to the 5-HT system in each

brain region.

Circadian Aspects

The effect of RU24969 showed a significant circadian variation in the hippocampus, frontal cortex and striatum but not in the hypothalamus. It appears strange that the hypothalamus, the seat of the SCN, displayed no circadian rhythm in sensitivity to autoreceptor stimulation. This might be because whole hypothalamus was used and the different nuclei in the preparation displayed differing rhythms that effectively cancelled each other out.

The variation in the response to RU24969 found in the 3 other brain regions could be due to four factors.

There may be a circadian rhythm in the sensitivity or number of 5-HT_{1B} autoreceptors in the hippocampus, frontal cortex and striatum. A significant increase in total 5-HT_{1B} binding during the dark phase has been reported in the SCN (Prosser et al. 1993) although these authors only compared total 5-HT_{1B} binding at one time point in the light phase and one time point in the dark phase. In my study the hypothalamic response was the same over the light-dark cycle although there was a trend towards more inhibition of 5-HTPacc at the end of the dark phase. In a more comprehensive study, 5-HT_{1B} binding was measured over 24 hours in the cerebral cortex of male Wistar rats (Akiyoshi et al. 1989). The number of 5-HT_{1B} binding sites was low throughout the dark period and rose after the lights were switched on to peak 3 hours after lights on, falling steadily through the light phase and reaching a minimum 6 hours into the dark phase. The results presented here show that the receptor appears more responsive during the light phase in the frontal cortex which would agree with the findings of Akiyoshi and colleagues i.e. maximal inhibition when the receptor number is maximal. There is also behavioural evidence to suggest a rhythm in 5-HT_{1B} function (Martin et al. 1987) although the behavioural model used is a measure of post-synaptic 5-HT_{1B} receptors. The authors found that 5-HT_{1B} function was greater during the light phase than the dark phase in mice. However Singh and Redfern (1994a) found no

difference in cortical autoreceptor function over 24 hours when measured *in vitro*. Binding studies performed over 24 hours could help to elucidate this point.

A circadian rhythm in the receptor-effector coupling mechanism could underlie the differences. A circadian rhythm in the basal concentration of cAMP has been demonstrated (Prosser and Gillette 1991). The variation is due to a circadian rhythm in the activity of the phosphodiesterase (PDE) not adenylate cyclase. Thus the inhibitory effect of stimulating the 5-HT autoreceptor could lead to a greater decrease in cAMP levels at some time points than others. However the results of experiments conducted by Lemmer and colleagues (1991) suggest that there is no rhythm in the induction of cAMP by β-adrenoceptor agonists. However, as the authors discuss, it is possible that there is a seasonal rhythms in adenylate cyclase activity with a circadian rhythm in activity only at distinct times of the year. This has been shown for β-adrenoceptors in rat heart ventricles which display a circadian rhythm in binding only during the autumn and winter. Equally PKA or Ca^{++}/CaM PKII activity or levels, or protein 14-3-3 might vary over 24 hours, although to date there are no studies measuring these parameters. Measurement of the inhibition of forskolin-stimulated AC by 5-HT_{1B} ligands, PKA or Ca^{++}/CaM PKII levels and activity over 24 hours would decide which one, or combination, of these factors could be responsible for the variation.

It is well established that 5-HT release is higher in the dark phase in the hippocampus (Kalen et al. 1989) and the hypothalamus (Martin and Marsden 1985; Imeri et al. 1994). However the levels do vary considerably within each phase of the light-dark cycle (Kalen et al. 1989). The same volume of RU24969 was administered at all four time points but if the biophase concentration of 5-HT was greater at some time points, then the effect of RU24969 might be additive. For example if more 5-HT was released at mid dark than mid light, then after RU24969 administration more 5-HT_{1B} agonist would be present in the synaptic cleft at mid dark to induce a response. Indeed the results support this theory to a certain extent; in the striatum and hippocampus 5-HT receptor function appeared greater during the light phase. Measurement of the 5-HT levels in each brain region over 24 hours by microdialysis would define this point.

Chronic Antidepressant Treatment

Basal TrOH activity in treated animals

Rats were treated chronically with two antidepressant drugs with differing effects. Desipramine is a selective noradrenaline uptake inhibitor (K_i NA 0.61nM, 5-HT 180nM and DA 11000nM; Bolden-Watson and Richelson 1993) and paroxetine a selective 5-HT uptake inhibitor (K_i NA 33nM, 5-HT 0.73nM and DA 1700nM; Bolden-Watson and Richelson 1993). Rats were treated with the antidepressant whilst they were being phase-shifted. This was necessary to ensure the rats were the same final weight as rats used for the initial RU24969 experiments for comparability. Another factor to be taken into consideration is age, since monoamine levels and 5-HT receptor numbers are known to decrease with age. Treatment with 5-HT and NA re-uptake inhibitors has been shown either to have no effect (Brown and Seggie 1988; Refinetti and Menaker 1993) or to increase (Baltzer and Weiskrantz 1975; Brown and Seggie 1988) the rate of re-entrainment to a shift in the light-dark cycle. There was one obvious drawback to the experimental protocol used. The time when drugs were administered could not be standardised because the rats might take the injection as a cue and entrain to it. Neither could the antidepressants be administered by drinking water or by osmotic minipumps since paroxetine will only dissolve (and stay dissolved) in warm water. Additionally osmotic minipumps could not be used since they would have to be replaced during the study and subjecting rats to anaesthesia could have interfered with the results. Consequently, rats were injected in a random fashion and some rats received injections solely during their active phase, others during their inactive phase and a third group during both phases. Thus some of the results might be related to specific timing of antidepressant administration, although to date there is very little in the literature about this aspect of pharmacology. A great advance could be made if programmable time release drug formulations could be made and a relationship between time of administration and clinical efficacy measured.

Chronic treatment with either antidepressant had no effect on basal TrOH activity when measured at end light. End light was chosen as a representative time

point because the RU24969-induced decreased in 5-HTPacc was greatest throughout the brain at this time. It might be argued that the attenuation of RU24969's effects on 5-HTPacc after chronic antidepressant treatment might not represent a "down-regulation" of the 5-HT_{1B} receptor; rather that chronic antidepressant treatment had increased TrOH activity as demonstrated by Moret and Briley (1992) or Redfern and Sinei (1986). Thus RU24969 would decrease 5-HTPacc less in antidepressant-treated rats than saline-treated control animals because there was more TrOH present. Both antidepressants, administered chronically, attenuated the effects of RU24969 in the hypothalamus and striatum at end light. However in the hippocampus only chronic desipramine caused "down-regulation", and in the frontal cortex neither antidepressant affected the decrease in 5-HTPacc mediated by RU24969. These facts, taken together with the finding that at end light there was no significant change in basal 5-HTPacc after chronic antidepressant treatment and "up-regulation" at some time points, argue against an induction of TrOH. In addition there are experimental differences between this study and those of Moret and Briley (1992) and Redfern and Sinei (1986). Moret and Briley treated Sprague-Dawley rats orally, twice daily with either 20mg/kg/day citalopram (a selective 5-HT uptake inhibitor) or milnacipram (a MAOI) and measured 5-HTPacc after AAAD inhibition by NSD1015 (100mg/kg i.p. 30 minutes before death). However their study was not a circadian one. Redfern and Sinei (1986) treated Wistar rats twice daily with either 20mg/kg i.p. mianserin (an atypical antidepressant which is thought to be an antagonist at 5-HT_2 receptors) or 7.5mg/kg clomipramine (a selective 5-HT uptake inhibitor) and measured 5-HT synthesis by the CO_2 trapping method at mid light and mid dark. Svensson (1978) found no change in basal 5-HT synthesis rate after chronic imipramine (a 5-HT/NA uptake inhibitor). Measurement of TrOH activity *in vivo* and the level of mRNA for TrOH after chronic antidepressant treatment over 24 hours would decide whether TrOH activity was increased or TrOH was induced or not after antidepressant treatment.

Chronic antidepressant treatment is complicated by our ignorance of its effects on all receptor populations. As outlined on page 24 of the introduction, the biophase

concentration of 5-HT is determined by whether somatodendritic and terminal autoreceptors down-regulate or not and the experimental evidence is contradictory. In addition the DR and locus coeruleus (LC) receive mutual innervation, and the two systems can affect the firing rate and release of the other (Clement et al. 1992b). The role of α_2-autoreceptors on NA terminals in the DR after chronic desipramine treatment, is therefore crucial since they control the biophase concentration of noradrenaline in the DR and thereby the tonic stimulatory effect of the α_1-adrenoheteroreceptor on the firing rate of DR neurons. After chronic desipramine treatment α_2-adrenoceptors normally down-regulate in a classical manner. Therefore the response of α_1-adrenoheteroreceptors to the increased biophase level of NA will also determine the rate of 5-HT neuronal firing. There is no experimental evidence of the effect of chronic antidepressant treatment on these α_1-adrenoheteroreceptors. However if these α_1-adrenoheteroreceptors do down-regulate it might not necessarily affect the firing rate of DR neurons because if they did down-regulate in response to increased NA levels, the decrease in 5-HT neuronal firing rate would decrease 5-HT release from dendrites and lessen 5-HT_{1A} autoreceptor stimulation. Thus the effect of down-regulation of α_1-adrenoheteroreceptors would be negated by decreased stimulation of inhibitory 5-HT_{1A} autoreceptors.

Regional Differences

Significant regional differences in the 5-HTPacc after RU24969 were detected after chronic treatment with both paroxetine and desipramine. Regional differences in receptor down-regulation after chronic antidepressant treatment have been noted before (Martin et al. 1992b).

These regional differences might represent differing regional "susceptibilities", i.e. 5-HT_{1B} receptors in some brain regions might be less sensitive to increases in the biophase concentration of 5-HT than other areas. Regional differences in 5-HT receptor populations after chronic antidepressant treatment are well documented (for example Martin et al. 1992b). This theory could be tested by implanting *in vivo*

microdialysis probes in the various brain region.

Alternatively the differences could be due to a mixture of preferential innervation from different RN nuclei and the heteroreceptors located somatodendritically. Earlier the proposal that there are two types of 5-HT_{1A} receptor was put forward. There is evidence that two different types of 5-HT_{1A} receptor are affected differently by repeated treatment with 8-OH-DPAT (Larsson et al. 1990), one type of receptor down-regulating after repeated treatment, the other remaining the same. The same might be true after chronic antidepressant treatment. The relative proportions of the two types of 5-HT_{1A} receptor on the cell body could affect the terminal release of 5-HT and thus the degree of feedback inhibition.

There are no experimental data on the effect that chronic antidepressant treatment has on cell body heteroreceptors. Each of the array of heteroreceptors might be phase-dependently down- or up-regulated thus affecting terminal 5-HT release.

An added dimension to the effect of chronic antidepressant treatment on autoreceptors and heteroreceptors is their topographical organisation. The proportion of these receptors on cell bodies and whether they down-regulate in subregions of the DR or MR may affect terminal 5-HT biophase differently depending on the brain region.

At the nerve terminal the only heteroreceptor to have been investigated after chronic antidepressant treatment is the a_2-heteroreceptor. It has been shown to be down-regulated (Gonzalez et al. 1992) or unchanged in the cortex and hippocampus (Schoeffelmeer et al. 1982), after chronic treatment with the same antidepressant, but for differing treatment times; Gonzalez and colleagues treated for 14 days whilst Schoefflmeer and co-workers treated for 28 days.

All of these parameters could be tested using *in vivo* microdialysis to assess somatodendritic and terminal autoreceptor and heteroreceptor function after chronic antidepressant treatment. The effect of microinfusion of ligands within subregions of the RN on terminal release might be used to assess topographical influence.

This is the first demonstration of down-regulation of 5-HT_{1B} autoreceptor

control of 5-HT synthesis *in vivo* following prolonged antidepressant treatment. The results indicate that control of synthesis is compromised following antidepressant treatment and that the compromise is regional.

Circadian Aspects

As shown in figure 31 to 34, the "rhythm" in RU24969-induced inhibition of 5-HTPacc was significantly altered in some brain regions by chronic antidepressant treatment. There are several possible explanations for how chronic antidepressant treatment changed the parameters of RU24969-mediated inhibition of 5-HTPacc with time. There are no published comparable studies to my knowledge.

Antidepressant treatment could be altering circadian parameters of serotonin function. Desipramine and paroxetine, like all other antidepressants, inhibit liver tryptophan pyrrolase (Badawy and Evans 1982; Badawy and Morgan 1991) and therefore increase circulating levels of l-try. This could interfere with the circadian rhythm in plasma l-try and thus l-try entry into the brain ultimately affecting 5-HT synthesis. Indeed Redfern and Martin (1985) have shown that chronic antidepressant treatment alters the acrophase of plasma l-try. In general antidepressant treatment phase delayed the peak in plasma l-try levels. Antidepressant treatment also modifies circadian parameters of 5-HT synthesis in the hypothalamus (Greco et al. 1988). Before chronic antidepressant treatment the levels of l-try, 5-HTP and 5-HT in hypothalamic homogenates were well correlated, all being higher at the transition from light to dark. Antidepressant treatment phase-delayed the position of the l-try, 5-HTP and 5-HIAA peaks, but phase-advanced the 5-HT peak. The mechanism by which antidepressant drugs achieve these effects is unclear. However it is unlikely that shifting the peak plasma l-try concentration could affect the circadian rhythm in brain 5-HT (see Morgan and Yndo 1973 and Liozou and Redfern 1986, as cited earlier in this discussion).

Prolonged antidepressant treatment could alter a circadian rhythm in presynaptic 5-HT_{1B} receptor number or affinity. Although, to date, the binding of

presynaptic 5-HT_{1B} has not been investigated, total 5-HT_{1B} binding in the cortex is increased during the light phase and lower throughout the dark phase (Akiyoshi et al. 1989). As outlined on page 28 of the introduction, a phase delay in the peak binding of α- and β-adrenoceptors, GABA-benzodiazepine and opiate receptors has been reported after chronic antidepressant treatment (Wirz-Justice et al. 1982). The rhythm of muscarinic acetylcholine receptors is also altered after antidepressant treatment (Kafka et al. 1981b; Wirz-Justice et al. 1982). Thus chronic antidepressant treatment can alter receptor numbers in a phase-dependent manner. How chronic antidepressant treatment might affect the expression of neurotransmitter receptors has not been investigated. It is possible that 5-HT_{1B} receptors might be able to control their own expression by regulating their transcription as has been proposed for β_2-adrenoceptors, which are also G protein linked receptors. A proposed mechanism is detailed on the left hand side of figure 36. Cyclic AMP response element-binding protein (CREB) is known to mediate the effects of cAMP on gene expression (Nestler and Greengard 1994), it binds to a specific sequence of DNA termed a cAMP response element (CRE) in the regulatory region of the gene and can influence the rate of transcription. It is thought that an increase in intracellular cAMP causes PKA to phosphorylate CREB, thus activating it and enhancing transcription. The CREB is also phosphorylated by Ca^{++}/CaM PKII. In the scenario outlined in an earlier section, stimulation of the 5-HT_{1B} receptor decreases the intracellular level of cAMP and Ca^{++} and thus PKA and Ca^{++}/CaM PKII activity thus decreasing the rate of transcription of e.g. 5-HT_{1B} receptors, given that there is a basal activity of adenylate cyclase. Regulation of transcription is a slower mechanism than receptor desensitisation and this could explain the delay in clinical efficacy witnessed.

Therefore repetitive stimulation of the autoreceptor might affect the normal 5-HT_{1B} control of the expression of 5-HT_{1B} receptors; this might explain why "up-regulation" was seen in this study as well as "down-regulation". The level of mRNA encoding 5-HT_{1A}, 5-HT_{1B} and 5-HT_2 receptors in whole brain (Spurlock et al. 1994), and 5-HT_2 receptors in the forebrain (Roth and Ciaranello 1991) has been found to be

the same after chronic treatment with either mianserin, imipramine, fluvoxamine or citalopram. However since these workers did not separate brain structures the overall changes in mRNA could have been cancelled out. Interestingly, incubating cultured raphe cells with cAMP for 48 hours decreases the mRNA for 5-HT_{1A} receptors but did not affect mRNA for 5-HT_2 receptors (Foguet et al. 1993), indicating a cAMP-dependent mechanism for modulating a receptor negatively coupled to adenylate cyclase. The mRNA for 5-HT_{1B} receptors and its regulation by PKA could be measured after chronic antidepressant treatment over 24 hours to assess this factor.

The phase-dependent up- and down-regulation of 5-HT_{1B} autoreceptor function seen after chronic desipramine and paroxetine may also be due to effects on other intracellular mechanisms or possibly the 5-HT_{1B} receptor-effector mechanism becomes uncoupled. Interestingly, the time course of intracellular modification again more closely parallels the delay seen in clinical efficacy of antidepressant drugs. Prolonged treatment with desipramine has been shown to modify the cAMP-dependent phosphorylation of microtubule-associated protein, which is involved in microtubule function e.g. transport of receptors and/or synthesising proteins to the nerve terminal (Perez et al. 1989). This may mean that chronic antidepressant treatment interferes with the transport of 5-HT_{1B} receptors to the nerve terminal, affecting the number of receptors in the terminal region.

It is feasible that 5-HT_{1B} receptor coupling to adenylate cyclase via its G_i protein is disrupted after chronic antidepressant treatment. Treatment with desipramine has been shown to decrease the accumulation of cAMP-induced by noradrenaline *in vitro* (Okada et al. 1988; Sapena et al. 1992); the effect has been linked to a decrease in coupling between the β-adrenoceptor and its G_s protein (Okada et al. 1988). Long-term treatment with imipramine, desipramine and clorgyline decreases the immunoreactivity and mRNA for G_i (Lesch et al. 1991b), although the effect was antidepressant and region specific. The activity of PKA is also altered after chronic antidepressant treatment (Nestler et al. 1989). The enzyme activity in the particulate fraction was decreased after antidepressant treatment, whilst activity was increased in

the crude nuclear fraction. The authors postulate that antidepressant treatment induces translocation of the catalytic subunits of PKA from the cytosol to the nucleus. As hypothesised earlier, the expression of 5-HT_{1B} receptors may be controlled by a mechanism involving PKA. Antidepressant-induced changes in PKA activity could therefore alter the rate of transcription of the 5-HT_{1B} receptor gene by an action at the CRE in the regulatory domain. Changes in various G proteins have been detected following chronic antidepressant treatment (Lesch et al. 1992). However, these results are very complicated. Both increases and decreases in G_s, G_i and G_o were found and the change was both antidepressant and regionally specific. For example, desipramine treatment decreased the α subunit of G_s in the striatum and LC and G_i in tjhe frontal cortex, whilst it increased the α subunit of G_o in the frontal cortex and LC. Therefore the only conclusion that can be drawn is that there are changes, but what actually happens to G proteins in different neurotransmitter systems is unclear.

Like the β_2-adrenoceptor, there are two PKA target sites on the 5-HT_{1B} receptor (Voigt et al. 1991), as outlined on page 10 of the introduction. If 5-HT_{1B} regulation of intracellular cAMP levels is lost PKA levels/activity might rise and phosphorylate these sites. Phosphorylation of the two PKA binding sites on the β_2-adrenoceptor is thought to be responsible for the decreased sensitivity of the receptor after chronic exposure to agonists (Hausdorff et al. 1989). Both PKA binding sites adjoin the region where the β_2-adrenoceptor interacts with its G_s protein. If the same were true of the 5-HT_{1B} receptor then chronic antidepressant treatment could affect 5-HT_{1B} function through PKA. This effect could be phase-dependent perhaps because of the circadian rhythm in intracellular cAMP levels.

Thus the intracellular mechanisms by which antidepressant treatment affects receptors could be linked to cAMP and PKA regulation of receptor expression or receptor transport or alternatively, changes in G proteins and coulping of the receptor to its G protein, by whayever mechanism.

The results of this section clearly demonstrate that chronic antidepressant treatment can affect 5-HT_{1B} receptor control of 5-HT synthesis in a time of day

dependent manner. Therefore the normal pattern of control of synthesis over 24 hours will be altered, perhaps leading to changes in the amount of 5-HT released per nerve impluse. This would in turn affect the 24 hour differences in stimulation of post-synaptic targets, altering 5-HT modulation of converging signals in that neuron. This effect of antidepressants on 5-HT_{1B} receptor-mediated control of synthesis might be part of the antidepressant's clinical efficacy.

Summary

5-HT synthesis through the light-dark cycle displayed a marked variation in the hypothalamus, hippocampus, frontal cortex and striatum. Administration of RU24969 decreased the production of 5-HTP and the receptor responsible was pharmacologically defined as a 5-HT_{1B} receptor. In the hippocampus, frontal cortex and striatum, inhibition of 5-HT synthesis varied significantly over 24 hours. However, each brain region displayed a characteristic pattern but, interestingly, the hypothalamus showed no variation. Chronic antidepressant treatment with either paroxetine or desipramine altered the effects of RU24969 on synthesis; both receptor down-regulation and up-regulation were observed. In effect prolonged antidepressant administration altered the 24 hour pattern of 5-HT_{1B} inhibition of 5-HT synthesis. The results are in agreement with the hypothesis.

Chapter 4 In vivo microdialysis

Hypothesis

In vivo microdialysis experiments tested the hypothesis that in entrained rats;

a) The function of the terminal autoreceptor differs at two time points in the light-dark cycle.

b) Chronic antidepressant treatment would significantly down-regulate autoreceptor function.

c) The degree of down-regulation at the two time points would be different.

The studies were performed *in vivo* in order to assess the 5-HT system when it was interacting with other neurotransmitter systems and while humoral factors were intact ; thus the parameter measured was physiologically relevant.

Method

The principle of microdialysis is simple. A microdialysis probe is stereotaxically implanted into a specified brain region. Low molecular weight compounds will diffuse across a semipermeable membrane driven by a concentration gradient from the extracellular fluid to the perfusing artificial cerebrospinal fluid. The dialysate can then be analysed for specific compounds. Page 33 of the techniques appraisal gives details of the principles of microdialysis.

The experiments described in this chapter were performed at Boots Pharmaceuticals Research Department, therefore some of the experimental details, including the HPLC apparatus, were different from those described earlier for the experiments performed at the University of Bath.

Implantation of the dialysis probe

Male Wistar rats (Olac, Bicester) weighing 240-260g were housed in pairs under a 12:12 light-dark cycle (lights on 0600), at an ambient temperature of 21°C and had free access to food and tap water.

Rats were anaesthetised with chloral hydrate (600mg/kg i.p.) and supplementary doses of anaesthetic (30mg i.p.) were given as needed. A rectal probe, attached to an homeothermic blanket (Model 50-7016, Harvard Instruments), was inserted to monitor core body temperature and maintain it at 36.8°C. Ear bars were inserted and the head of the anaesthetised rat placed in a stereotaxic frame with the incisor bar set at -3.3mm, to ensure a flat skull (David Kopf Instruments). A vertical scalpel cut the length of the skull was made and the loose skin kept clear of the skull by artery clips. The skull surface was scraped clean of any tissue and the skull cauterised with hydrogen peroxide to expose Bregma more clearly. The dialysis probe (CMA/12 microdialysis probe, 2mm long membrane and 0.5mm o.d., Carnegie Medicin, Biotech Instruments) was positioned directly above Bregma and then lowered slowly so the tip just touched the skull surface, the position of the probe was checked under microscope (Nikon SMZ-1) and the co-ordinates for Bregma noted. The co-ordinates for the anterior hypothalamus were calculated with reference to Bregma and the skull surface (anterior-posterior (AP) -1.3, mediolateral (ML) -0.6 and depth -9.3) according to the atlas of Paxinos and Watson (1982). The position was marked and a circular burr hole made with an electric drill (RS Instruments) around the mark almost through the skull surface. The passage of the probe lies adjacent to the sagittal sinus, so the bone was gently removed from the opposite side from the sinus to avoid piercing the sinus and promoting gross bleeding. If the sinus was nicked, any bleeding was stopped using saline-soaked cotton wool and gentle pressure. The dura was pierced with a 22 gauge needle and the microdialysis probe was slowly lowered to the required depth. The exposed brain was covered with cotton wool soaked with saline to prevent drying and the skin pulled back across the skull. Figure 37 is taken from the atlas of Paxinos and Watson (1982) to show the final position and relative size of the microdialysis probe in the rat brain.

The probe was continuously perfused at a rate of 1µl/min (model 22 Microinjection pump, Harvard Apparatus or CMA/100 Microinjection pump, Carnegie Medicin, Biotech Instruments) with artificial cerebrospinal fluid (aCSF) (composition

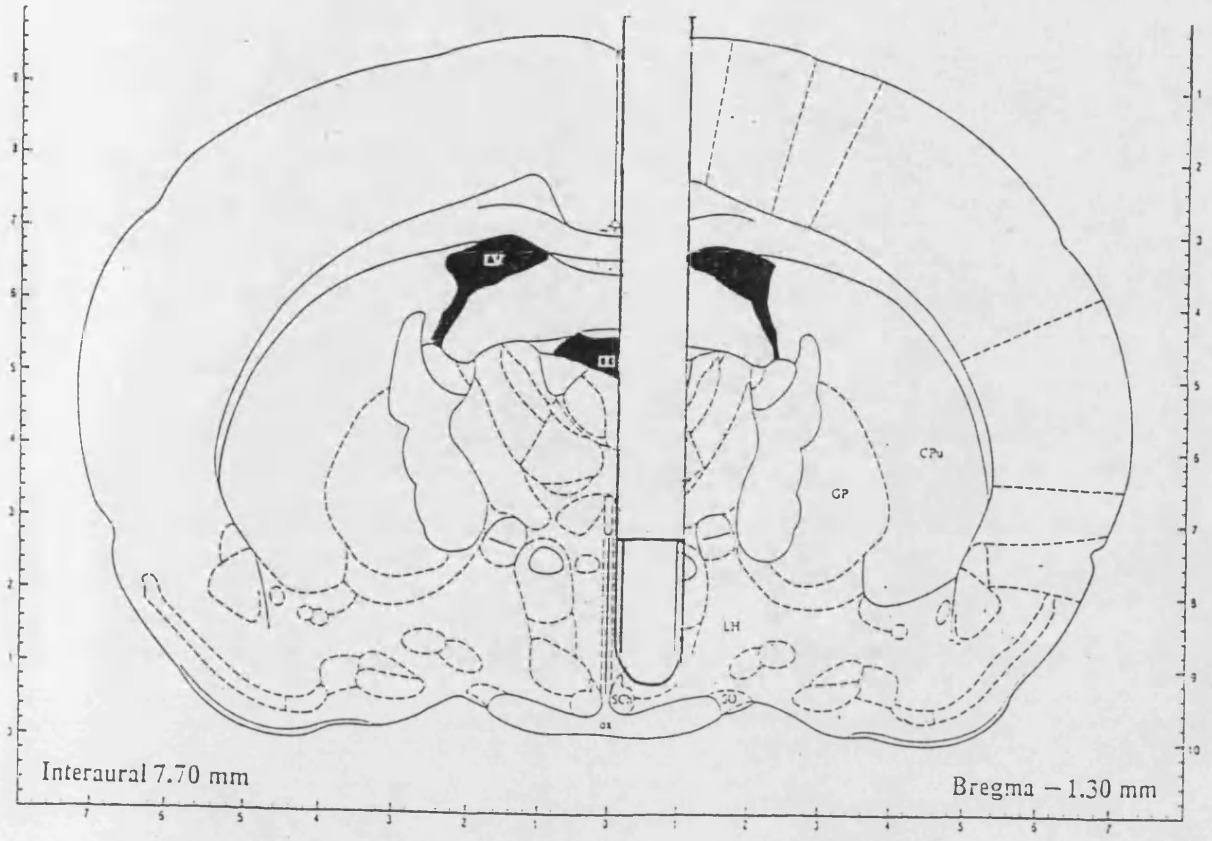


Figure 37. Coronal section through the rat brain, at the -1.3mm from Bregma level, to show where the final position of the microdialysis probe would be. Each major tick horizontally and vertically represents 1mm, the probe was implanted -0.6mm from 0 mediolaterally and -9.3mm deep. The probe is 0.5mm wide and had a dialysis membrane 2mm long. It can therefore be seen that 5-HT in the dialysate originated from the anterior hypothalamus, suprachiasmatic nucleus and medial preoptic area. The cross-section is taken from the atlas of Paxinos and Watson (1982). D3V dorsal third ventricle; LV lateral ventricle, CPu caudate putamen; GP globus pallidus, AH anterior hypothalamus; LA lateral anterior hypothalamus, LH lateral hypothalamus; SCh suprachiasmatic nucleus, ox optic tract and SO supraoptic nucleus .

in mM: NaCl 147; KCl 4; $CaCl_2$ 4) containing the selective 5-HT uptake inhibitor citalopram (1μM), and the effluent collected onto ice. At the end of the experiment the brain was removed and the position of the probe was visually confirmed.

Experimental Protocol

At the beginning of each day the recovery of 5-HT from the probe in the dialysis solution was checked *in vitro* at the flow rate used. Probes were dialysed in aCSF containing 100nM 5-HT for 30 mins and the peak height compared to the standard of the same concentration. Probes with recoveries of below 15% were discarded. New probes were flushed through with aCSF, at a rate of 30μl/mins, before use. The dialysis bag at the tip of the probe was checked for air bubbles, which were removed by gently tapping the steel cannula of the probe.

No dialysate samples were collected from rats during the 90 mins after probe implantation. This was to allow the efflux of 5-HT to stabilise following the damage caused by implantation of the probe. Experiments performed previously in the laboratory have shown that this time is adequate. Two 15 mins aliquots were taken to serve as pre-intervention controls and successive 15 mins fractions collected throughout the experiment. For agonist studies the drug was infused via the probe for 15 mins, immediately after the control samples, and 6 subsequent samples were collected. For antagonist studies, the antagonist alone was infused via the probe for 15 mins, after the control samples, then the agonist and the antagonist were infused together via the probe for a further 15 mins. Dialysate samples were assayed for their 5-HT content using reverse phase high performance liquid chromatography coupled to electrochemical detection (HPLC-ECD), the principles of which are set out on page 36 of the techniques appraisal.

At the end of the experiment the dialysis probe was flushed through with distilled water for 30 mins, at a rate of 30μl/min, to prevent any buffer crystallising and subsequently blocking the tubing.

Measurement of 5-HT in the dialysate

The HPLC-ECD system consisted of a Hypersil ODS2 column (10 cm x 2 mm o.d.) with 3µm packing (HPLC Technology) linked to a Coulochem electrochemical detector with dual electrodes, electrode 1 set at +0.1V and electrode 2 at +0.28V (model 5011 analytical cell, ESA Inc.). Buffer (composition: NaH_2PO_4 0.1M; sodium octane sulphonic acid 0.93M; 0.07% v/v dibutylamine and 12% v/v methanol, adjusted to pH 3.0 with orthophosphoric acid) was supplied to the system by a Severn Analytical solvent delivery system (SA6410B Severn Analytical) at a flow rate of 0.45ml/min. Samples were injected, using a 20µl Hamilton syringe, into a Rheodyne (model 7125) injection port attached to a 20µl loop, the loop was flushed through with distilled water before each injection. The chromatogram was plotted on a Shimadzu C-R6A chromatopac integrator (Dyson Instruments Ltd.). The pump flow rate was decreased to 10µl/min overnight.

Chronic antidepressant treatment

Rats were treated for 21 days with a once daily injection of either desipramine hydrochloride (10mg/kg i.p.) or paroxetine hydrochloride (10mg/kg i.p.) or an equivalent volume of saline. Both rats in the cage received the same treatment, but all rats used in this study were housed in the same room and therefore under the same environmental conditions. The precise time of injection was varied each day to avoid the animals taking the injection as a time cue, but injection was always performed in the afternoon during the rat's light phase. All experiments were performed after a 24 hour washout period. One saline-treated animal and one antidepressant-treated animal were dialysed at the same time at each time point.

Drugs

All drugs infused via the probe were made up in aCSF including 1µM citalopram on the day of use. Chloral hydrate was made up in 0.9% sterile saline every 2 days as required.

Drug Suppliers

Drugs were purchased from suppliers as follows: NaCl, KCl, orthophosphoric acid and NaH_2PO_4 all Aristar quality (BDH Chemicals), octane sulphonic acid (Kodak Clinical Drugs Ltd.), HPLC grade methanol (Rathburn Chemicals), dibutlyamine (Aldrich), chloral hydrate, 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) and desipramine (Sigma Chemical Co.).

The following drugs were donated by the companies indicated; RU24969 (Roussel-Uclaf), citalopram (Lundbeck), methiothepin (Hoffmann-LaRoche) and paroxetine (SmithKline Beecham).

Statistics

Values are expressed as a percentage of the control value i.e. average concentration of 5-HT in the two dialysate samples taken immediately prior to any drug infusion. Data was analysed by two-way analysis of variance (ANOVA) to detect differences between treatments, followed by Studentised range test to determine where differences lay between treatments; $P \leq 0.05$ was considered significant. Statistical methods and outcomes are presented in appendix 1.

Results

All experiments were performed during the early to middle stages of the light period unless otherwise stated. Absolute values for 5-HT levels in the dialysate are given as a guide and are therefore not corrected for individual probe recoveries.

5-HT levels in control animals

The output of 5-HT into the dialysate was stable over the collection period. The mean 5-HT level in the first 30 mins of collection was 2.5±0.2fmoles/15µl sample. In general a control animal and a treated animal were dialysed at the same time, but for convenience all control values were pooled.

Calcium-dependence

The average 5-HT level in the first 30 mins of collection was 23±5.7fmoles/15µl sample. Calcium ions were removed from the perfusing aCSF for 60 mins, within the first 15 mins of this infusion there was a rapid decrease in 5-HT output, reaching a maximum decrease of 98% (2%±0.6%, $P<0.01$, n=4) at t=45 mins. When calcium ions were re-introduced, 5-HT levels increased rapidly back to baseline, increasing to a maximum of 121 ±6.6% at t=150 mins, figure 38.

$K^{\pm}$-dependence

Modified aCSF containing 100mM K^+ was infused via the probe for 15 mins. Pre-intervention 5-HT levels were 23±0.3fmoles/15µl sample. Infusion of 100mMK^+ led to an immediate and significant increase in 5-HT output of 456±11.5% ($P<0.01$, n=7) in the first fraction and 345±8.0% ($P<0.01$, n=6) in the second fraction, data not shown.

Infusion of 8-OH-DPAT (1µM)

8-OH-DPAT is a selective agonist at the 5-HT_{1A} receptor. It was used because subsequent experiments used RU24969 which is not completely selective for the 5-

HT_{1B} receptor, having an almost equal affinity for the 5-HT_{1A} receptor. 8-OH-DPAT was infused into the anterior hypothalamus to ensure that any effect of RU24969 produced was due to its activity as a 5-HT_{1B} agonist. Pre-intervention 5-HT levels were 34±4.5fmoles/15µl sample. A 15 mins infusion of 1µM 8-OH-DPAT had no significant effect on 5-HT release compared to the control animals, see figure 39.

Infusion of RU24969 (0.1-10µM)

Increasing concentrations of RU24969 were infused for 15 mins via the probe leading to a dose-dependent decrease in 5-HT output. Basal, pre-intervention 5-HT concentrations in the dialysis were 2.3±0.26fmoles/15µl sample in animals treated with 10µM RU24969, 3±0.4fmoles/15µl sample in rats infused with 1µM RU24969 and 1.6±0.18fmoles/15µl sample in animals receiving 0.1µM RU24969. At a concentration of 10µM, there was a maximum inhibition of 65% (35±2% at t=30 mins, $P<0.01$, n=6), 1µM 56% (44±1.7% at t=45 mins, $P<0.01$, n=4) and 0.1µM had a maximum inhibition of 49% (51±6.2% at t=45 mins, $P<0.05$, n=4), see figure 40.

Effect of Methiothepin on the response to RU24969

Methiothepin (metitepin) is a 5-HT_1 receptor antagonist; there are no selective 5-HT_{1B} antagonists. Methiothepin (1µM) was infused for 15 mins alone and then with 1µM RU24969 for 15 mins. Pre-intervention 5-HT levels were 17±1.6fmoles/15µl sample. Methiothepin reversed the RU24969-induced decrease in 5-HT output. In the presence of 1µM methiothepin, 1µM RU24969 caused a maximum inhibition of 5-HT output of 27% (73±7% at t=30 mins, n=4), which was not significantly different from the control animals. Methiothepin (10µM) also antagonised the effect of 5µM RU24969; pre-intervention 5-HT concentrations were 40±6fmoles/15µl sample, so that the maximal maximum inhibition of 5-HT output was 17% (83±3.8% at t=30 mins, n=4), which was not significantly different from controls.

Infusion of Methiothepin (1 and 10μM)

Basal 5-HT levels were 14±0.37fmoles/15μl sample. Infusion of methiothepin (1μM) alone for 30 mins significantly increased 5-HT output, with a maximum increase of 115 ±5.8% at t=45 mins ($P<0.05$, n=4). Pre-intervention 5-HT levels were 48±7.8fmoles/15μl sample. Infusion of methiothepin (10μM) alone also significantly increased dialysate 5-HT levels by a maximum of 242±7.1% at t=60 mins ($P<0.01$, n=5), see figures 41 and 42.

Basal 5-HT levels at mid light and end light

Basal 5-HT levels at mid light were 38.7±0.3fmoles/15μl sample (n=4). Basal levels of 5-HT at end light were 28.4±0.1fmoles/15μl sample (n=6) and there was no significant change in 5-HT release after lights off. The level of 5-HT in dialysate was significantly higher at mid light than end light ($p<0.05$ unpaired Student's t-test).

Infusion of RU24969 at mid light

There was a stable release of 5-HT from control animals over the course of the experiment and pre-intervention 5-HT levels were 41±4.6fmoles/15μl sample. When 5μM RU24969 was infused at mid light, i.e. 1200hr, it produced a maximal decrease in 5-HT release of 65% (35±3.3% at t=45 mins, $P<0.01$, n=4), with levels returning to basal by t=90 mins, see figure 43.

Infusion of RU24969 at end light

Basal 5-HT concentrations in the first 30 mins of collection were 35±2.2fmoles/15μl sample. Infusion of RU24969 at 1800hr, the time lights went off in the animal colony, significantly reduced 5-HT output by a maximum of 100 ±0% from t=60 mins ($P<0.01$, n=5), but, in contrast to mid light, 5-HT levels did not return to basal values over the course of the experiment, see figure 44.

Basal 5-HT levels in dialysate at mid light and end light in treated animals

Chronic antidepressant treatment did not affect basal levels of 5-HT (saline-treated 39±8.2fmoles/15µl sample, paroxetine 30.7±4.1fmoles/15µl sample, desipramine 34.4±2.2fmoles/15µl sample) measured at mid light. When measured at end light prolonged paroxetine treatment did not affect basal 5-HT levels but desipramine treatment increased basal levels compared to saline-treated animals (saline-treated 35±9fmoles/15µl sample, paroxetine 34.2±4.5fmoles/15µl sample, desipramine 41.3±5.6fmoles/15µl sample; one-way ANOVA with *post hoc* Studentised range test, $P<0.05$, n=4-5).

Effect of RU24969 at mid light in antidepressant-treated animals

RU24969 (5µM) infused in rats treated chronically with saline produced maximal inhibition of 51% (49±1.7% at t=60 mins, n=4). However when rats were treated with either of the two antidepressant drugs (paroxetine or desipramine), the effects of RU24969 were significantly attenuated from t=75 mins. Thus although the rate of onset and magnitude of the effect of RU24969 were not altered, the rate at which 5-HT levels returned to baseline was increased. At this time there was a rapid reversal of the effects of RU24969 in rats treated chronically with paroxetine or desipramine. Infusion of RU24969 via the dialysis probe in desipramine treated animals lead to a maximal inhibition of 47% (53±1.7% at t=45 mins, n=5). Paroxetine treated animals gave a similar result; with a maximal inhibition of 46% (54±2.9% at t=60 mins, n=4), see figure 45.

Effect of RU24969 at end light in antidepressant-treated animals

Infusion of RU24969 (5µM) via the probe decreased 5-HT output significantly in saline-treated rats, with a maximal inhibition of 98.8% (1.2±1.8%, n=4). Chronic antidepressant treatment significantly attenuated the effects of a 5µM probe infusion of RU24969. The maximal inhibition of 5-HT output in desipramine-treated rats was 35% (65±2.0% at t=15 mins, $p<0.01$, n=4), whereas paroxetine treatment led to a maximum

decrease in 5-HT release of 66.5% (43.5±1.9% at t=45 mins, $p<0.01$, n=5), see figure 46. Thus, at end light the rate of onset and the magnitude of the response to RU24969 were significantly decreased after chronic antidepressant treatment.

The degree and extent of inhibition of 5-HT output by RU24969 at the two time points after chronic antidepressant treatment were significantly different (one-way ANOVA with *post hoc* Studentised range test, $P<0.05$ n=4-5). However the statistical difference was found to stem from the rebound in dialysate 5-HT level in antidepressant treated rats at mid light from t=75mins. Thus chronic antidepressant treatment made the curves at mid light and end light after chronic paroxetine or desipramine the same, except for this rebound.

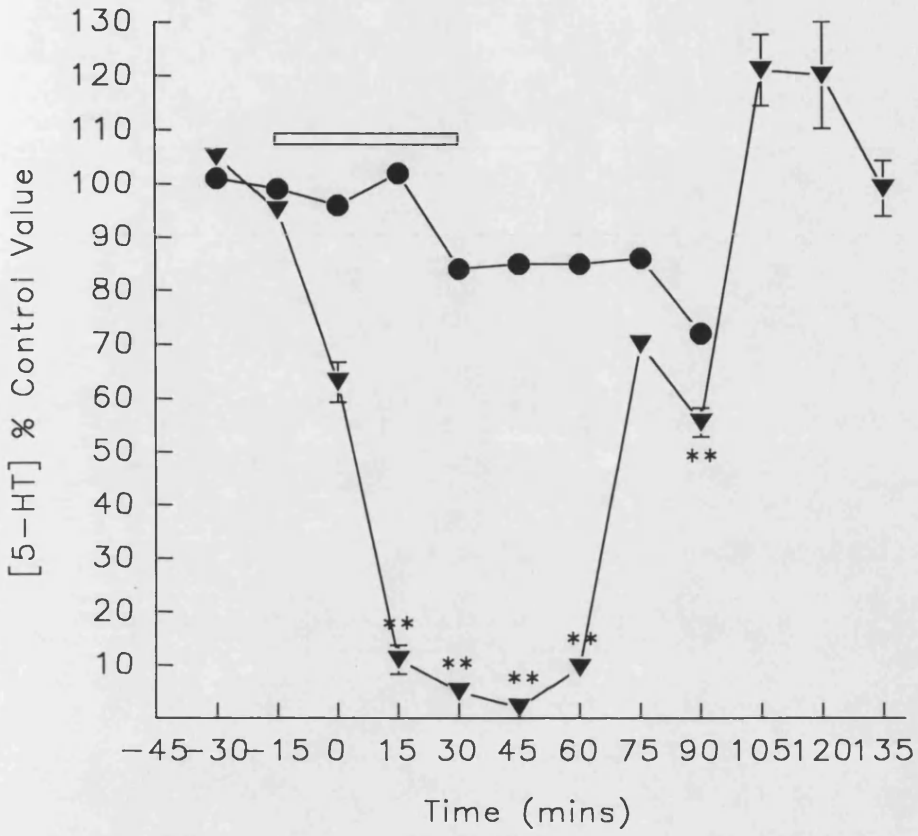


Figure 38. Effect of removing calcium ions from the perfusing medium on the level of 5-HT in the dialysate. Open bar indicates infusion of calcium-free buffer, which started at t=15 mins. Values are expressed as a percentage of the concentration of 5-HT in the two dialysate samples taken immediately prior to any intervention (control value), as mean ±s.e.m.,**P<0.01 vs pooled control, n=12 for pooled control and n=4 for treatment, pooled control (●) and Ca^{++} free buffer (▼).

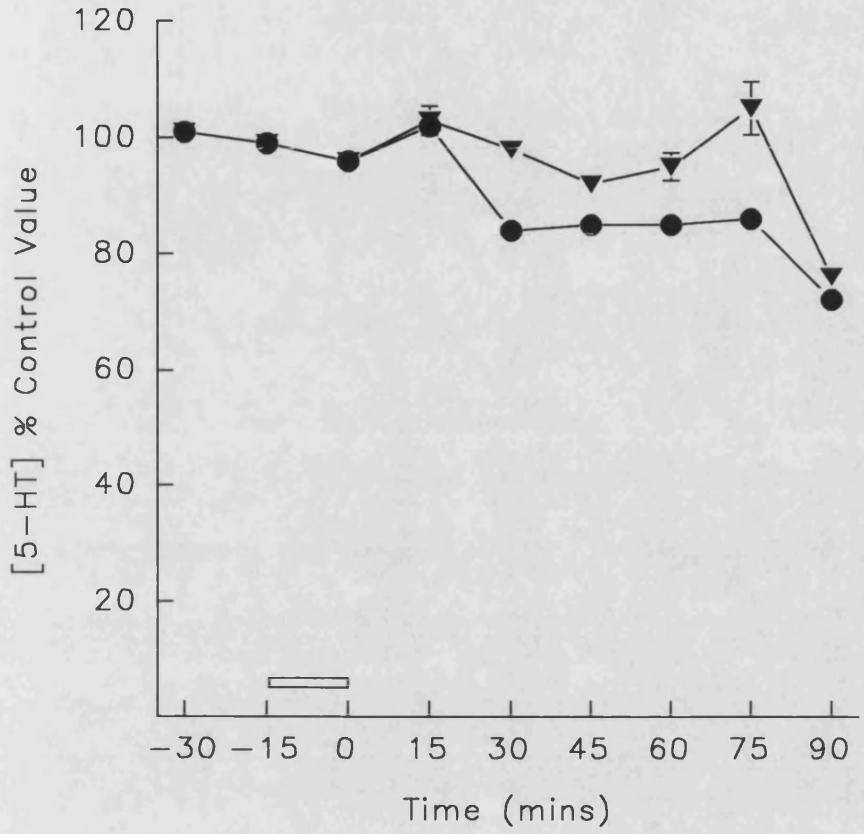


Figure 39. Effect of infusion of the 5-HT_{1A} agonist, 8-OH-DPAT, indicated by the open bar, on 5-HT output. Values are expressed as a percentage of the concentration of 5-HT in the two dialysate samples taken immediately prior to any intervention (control value), as mean ±s.e.m., n=12 for pooled control and n=4 for treatment, pooled control (●) and 1μM 8-OH-DPAT (▼).

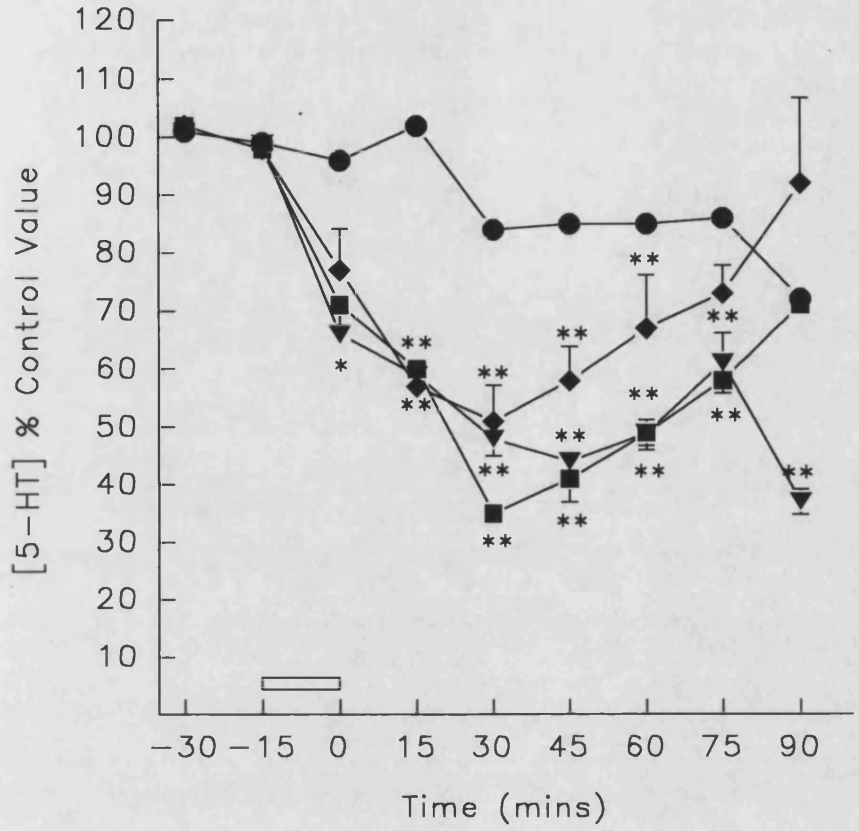


Figure 40. RU24969 (0.1μM, 1μM and 10μM) was infused for 15 mins, as indicated by the bar. Values are expressed as a percentage of the concentration of 5-HT in the two dialysate samples taken immediately prior to any intervention (control value), as mean ±s.e.m.,*P<0.05, **P<0.01 vs pooled control value, n=12 for pooled control, n=4 for 0.1μM and 1μM RU24969 and n=6 for 10μM RU24969, pooled control (●), 0.1μM RU24969 (◆), 1μM RU24969 (▼) and 10μM (■).

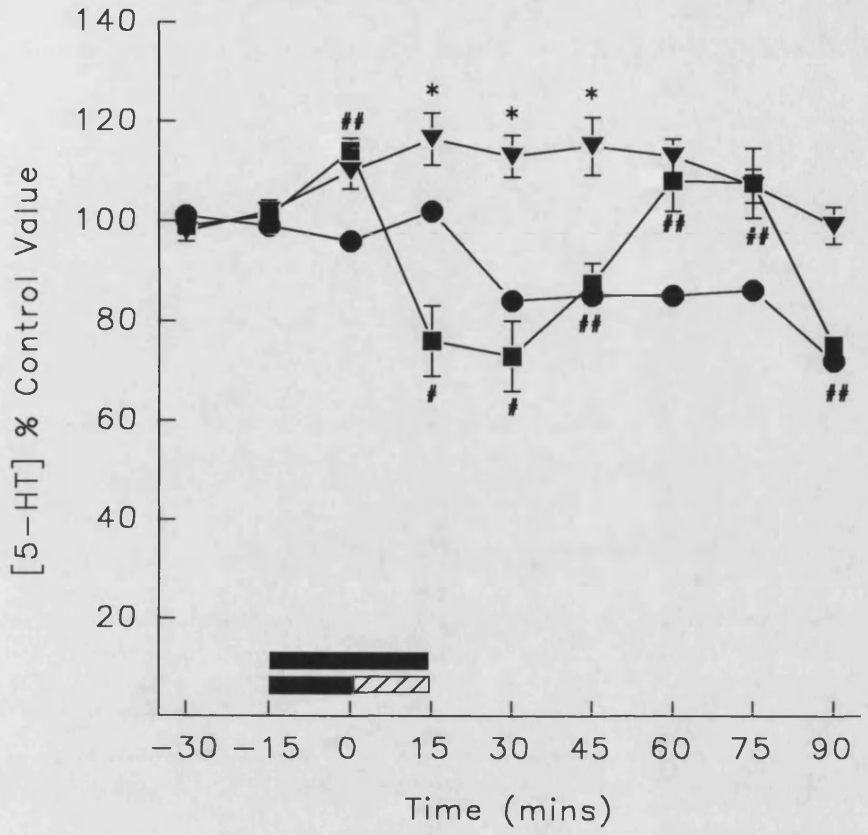


Figure 41. Effect of methiothepin infusion on the effect of RU24969. Methiothepin was infused for 15 mins, filled bar, and then either co-infused with RU24969 for a further 15 mins, stripped bar or infused alone, filled bar. Values are expressed as a percentage of the concentration of 5-HT in the two dialysate samples taken immediately prior to any intervention (control value), as mean ± s.e.m.,*P<0.05 vs pooled control value, $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs 1μM RU24969, n=12 for pooled control, n=4 for 1μM methiothepin and 1μM methiothepin + 1μM RU24969, pooled control (●), 1μM methiothepin + 1μM RU24969 (■) and 1μM methiothepin (▼).

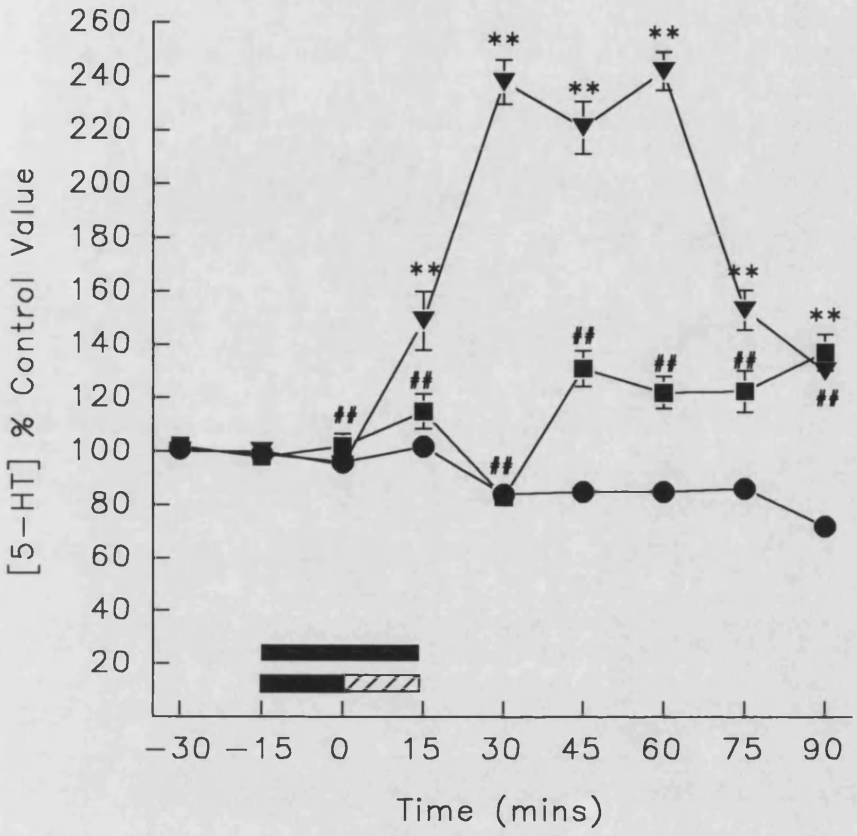


Figure 42. Effect of methiothepin infusion on the effect of RU24969. Methiothepin was infused for 15 mins, filled bar, and then either co-infused with RU24969 for a further 15 mins, stripped bar or infused alone, filled bar. Values are expressed as a percentage of the concentration of 5-HT in the two dialysate samples taken immediately prior to any intervention (control value), as mean ± s.e.m.,**P<0.01 vs pooled control value, $^{\#\#}$P<0.01 vs 5μM RU24969, n=12 for pooled control, n=5 for 10μM methiothepin and n=4 for 10μM methiothepin + 5μM RU24969, pooled control (●), 10μM methiothepin + 5μM RU24969 (■) and 10μM methiothepin (▼).

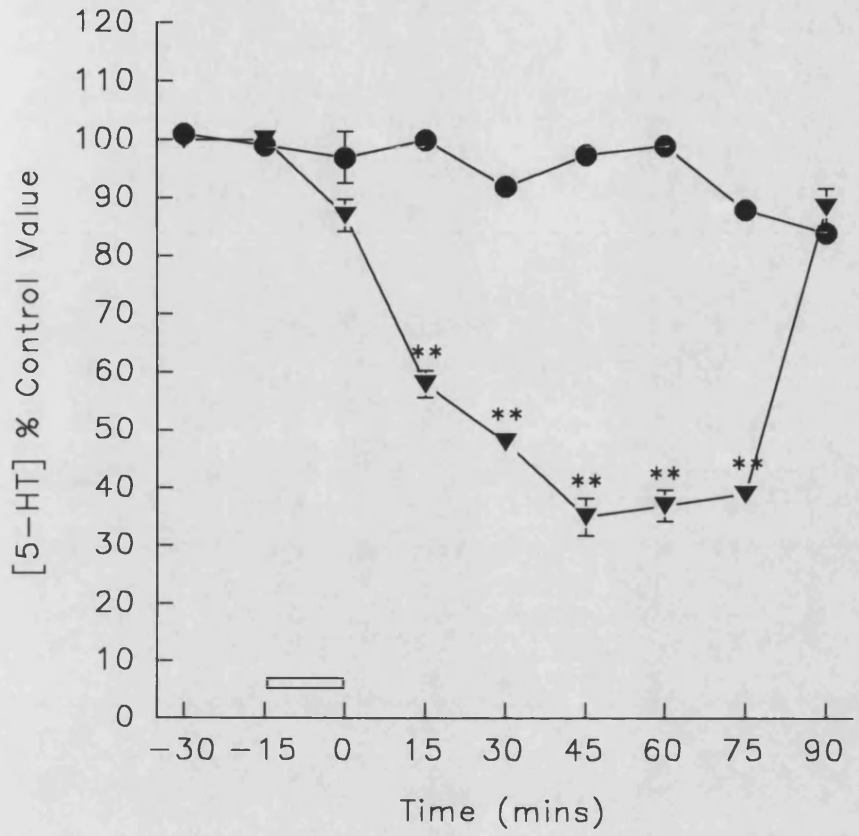


Figure 43. Infusion of 5μM RU24969, open bar, at mid light. Values are expressed as a percentage of the concentration of 5-HT in the two dialysate samples taken immediately prior to any intervention (control value), as mean ±s.e.m.,**P<0.01 vs control value for mid light, n=6 for mid light controls and n=4 for 5μM RU24969, control (●), 5μM RU24969 (▼).

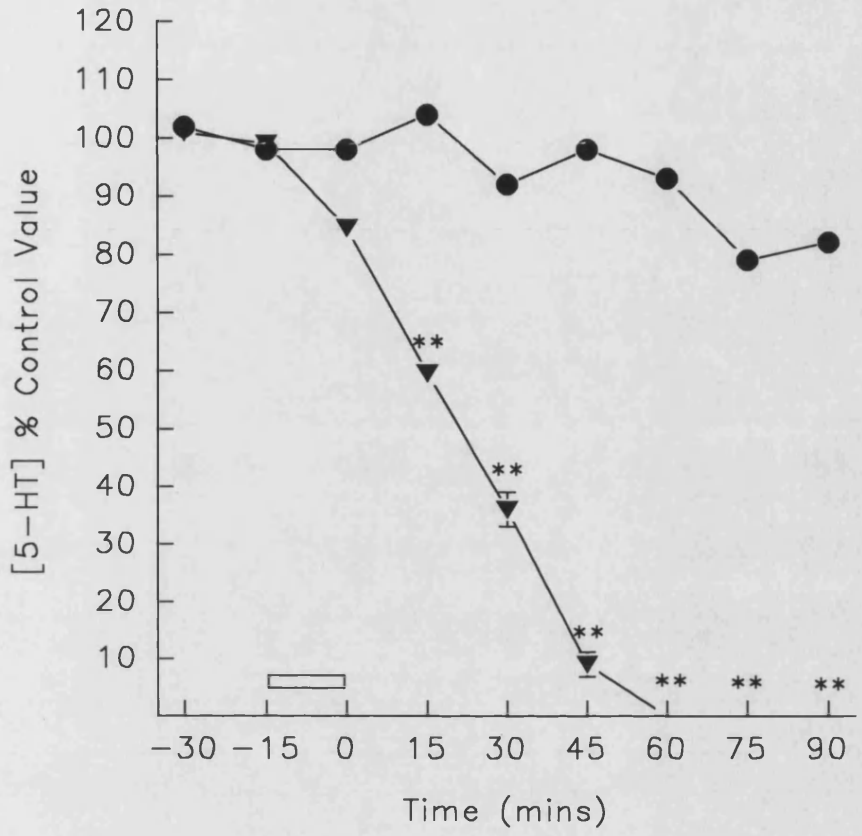


Figure 44. Infusion of 5μM RU24969, open bar, at end light. Values are expressed as a percentage of the concentration of 5-HT in the two dialysate samples taken immediately prior to any intervention (control value), as mean ±s.e.m.,**P<0.01 vs control value for end light, n=5 for end light controls and n=4 for 5μM RU24969, control (●), 5μM RU24969 (▼).

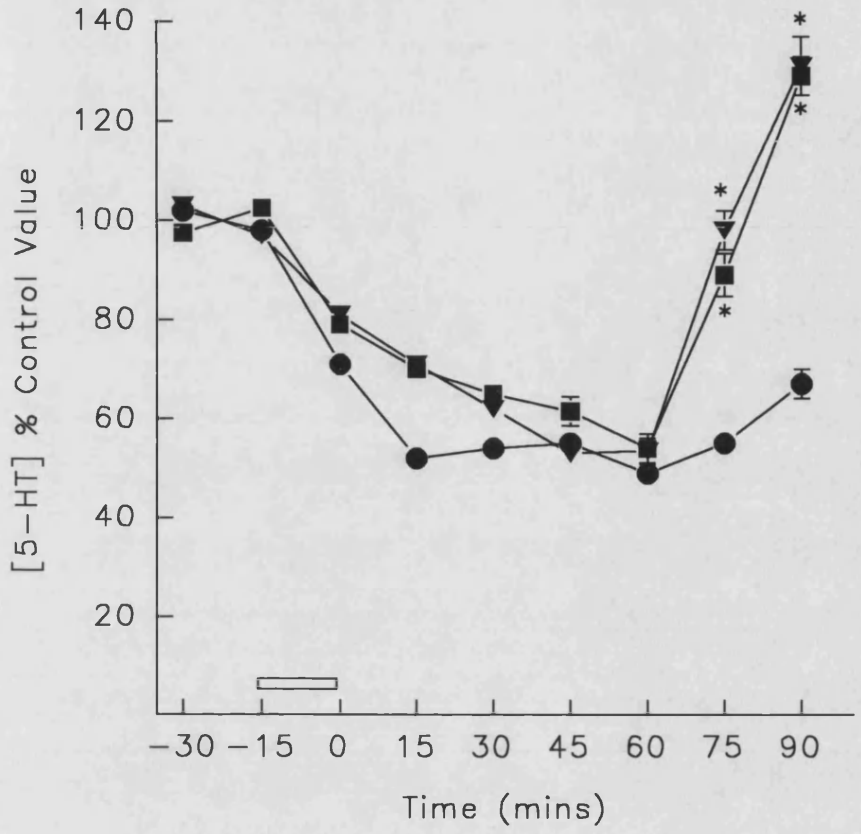


Figure 45. Rats were treated chronically with either paroxetine, desipramine or saline and the effect of 5μM RU24969, open bar, re-assessed at mid light. Values are expressed as a percentage of the concentration of 5-HT in the two dialysate samples taken immediately prior to any intervention (control value), as mean ±s.e.m.,*P<0.05 vs saline-treated rats, n=4 for saline-treated rats, n=4 for paroxetine-treated rats and n=5 for desipramine-treated rats, saline-treated + 5μM RU24969 (●), paroxetine-treated + 5μM RU24969 (■) and desipramine-treated + 5μM RU24969 (▼).

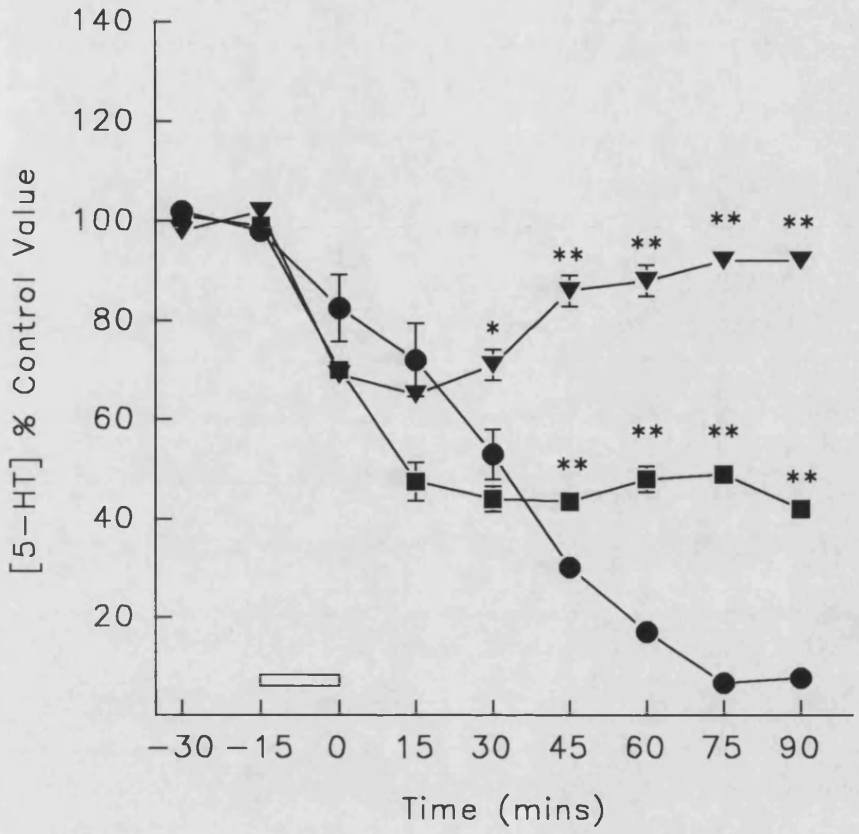


Figure 46. Rats were treated chronically with either paroxetine, desipramine or saline and the effect of 5μM RU24969, open bar, re-assessed at end light. Values are expressed as a percentage of the concentration of 5-HT in the two dialysate samples taken immediately prior to any intervention (control value), as mean ±s.e.m.,*P<0.05 vs saline-treated rats, n=6 for saline-treated rats, n=4 for both paroxetine-treated rats and desipramine-treated rats, saline-treated + 5μM RU24969 (●), paroxetine-treated + 5μM RU24969 (■) and desipramine-treated + 5μM RU24969 (▼).

Discussion

In vivo microdialysis was used to examine terminal 5-HT autoreceptor function in the hypothalamus of the anaesthetised rat, at two time points, after chronic antidepressant treatment. The hypothesis described earlier in this chapter predicted that the effects of chronic antidepressant treatment on 5-HT_{1B} autoreceptor function would be different at the two time points investigated in this study. The results presented here concur with this prediction.

The microdialysis probe was implanted into the anterior hypothalamus because this region is adjacent to the suprachiasmatic nucleus (SCN) and, like the SCN, it receives innervation from both RN. In fact, the microdialysis probe was implanted so that the medial side of the probe was located on the lateral border of the SCN. Thus at least a part of the dialysate 5-HT was likely to be derived from neurons in the SCN. It was not possible to implant the dialysis probe used here directly into the SCN because the SCN is too small; being 0.95mm long, 0.42mm wide and 0.40mm in depth, whilst the outer diameter of the probe is 0.5mm. However, other workers have implanted carbon fibre voltammetry electrodes combined with differential pulse voltammetry (8μm o.d.; Faradji et al. 1983; Martin and Marsden 1985) or specially constructed dialysis probes (o.d. 230μm; Glass et al. 1992) into the SCN and successfully measured 5-HT and 5-HIAA directly.

In order to remove the possible influence of environmental factors on 5-HT neuronal firing, it was decided to perform these experiments on anaesthetised animals. Sound and light have been shown to increase DR firing rate in freely moving cats (Heym et al. 1984). However, this increase is abolished if the animals were anaesthetised with chloral hydrate. On the other hand, chloral hydrate anaesthesia has been shown to decrease the activity of TrOH in rats (Tappaz and Pujol 1980) and to decrease the rate of DR neuronal firing in cats (Heym et al. 1984). In addition, since the 5-HT level was to be measured at end light, anaesthesia would avoid the influence of altered 5-HT release due to the rats starting to explore and groom during the dark phase. Although 5-HT release in the hippocampus does not reflect behavioural activity

state (Kalen et al. 1989), in the hypothalamus Imeri and colleagues (1994) have reported a close relationship between 5-HT release and arousal.

Origin of 5-HT Measured in the Dialysate

Sharp and colleagues (1989) have characterised 5-HT output in the hippocampus of anaesthetised rats, using a similar experimental protocol. These workers found that pre-treatment with the selective 5-HT neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT) given intraventricularly, decreased the 5-HT level in the dialystae by 57%. This indicates that at least half the 5-HT measured in the dialysate was derived from 5-HT neurons projecting from the DR, given that 5,7-DHT lesion only destroys DR neurons. Local infusion of tetrodotoxin into the hippocampus via the dialysis probe also decreased 5-HT levels by 75% testifying that most of the 5-HT measured was neuronally derived. Electrical stimulation of the the DR at frequencies of 2-20Hz i.e. encompassing the normal physiological firing rates, increased 5-HT output by a maximum of 400% at the highest frequency. There was no increase in 5-HT output when the stimulating electrode was implanted dorsal or ventral to the DR. These two results demonstrate that the 5-HT detected in dialystae is derived from 5-HT neurons.

The basal level of dialysate 5-HT measured in this study ranged from 1.6fmoles/15µl sample to 48fmoles/µl sample. These levels are lower than those reported by Sharp and colleagues (1989) of 100fmoles/20µl sample and by Kalen and co-workers (1988) of 20pmoles/60µl sample in the hippocampus but are close to those of Auerbach and workers (1989) of 5-8fmoles/60µl sample in the hypothalamus. Thus despite small differences, probably due to a number of factors: brain regions, longer interval between sample collection and different probe types (outer diameters and dialysis membrane length), as discussed in the techniques appraisal, the dialsyate 5-HT levels measured here are not dissimilar from those previously reorted (Kalen et al. 1988; Sharp et al. 1988; Auerbach et al. 1989).

Two methods were employed to verify that the overflow of 5-HT in the

dialysate was neuronally derived. First, since exocytotic release of neurotransmitters is considered to be calcium dependent, calcium ions were removed from the perfusate. Following this intervention, there was an almost immediate drop (98% decrease compared to pre-intervention values) in the levels of 5-HT, figure 38. When calcium ions were re-introduced into the aCSF, the concentration of 5-HT increased rapidly towards control values, with an increase in 5-HT levels above baseline by about 20% at the end of the experiment. Secondly, infusion of aCSF containing 100mM K^+ for 15 min caused an immediate statistically significant increase (465% compared to pre-intervention levels) in the overflow of 5-HT into the perfusing medium. Increasing the extracellular concentration of K^+ leads to depolarisation of the nerve membrane and concomitant release of neurotransmitter, similar to that seen when an action potential travels down the nerve and invades the nerve terminal. This K^+-dependence was further evidence that the 5-HT output measured in dialysate effluent was neuronally derived. Both these findings are in agreement with those of Auerbach and colleagues (1989) who have characterised dialysate 5-HT output in the hypothalamus of freely moving rats and found that the extracellular levels measured reflect neuronal release. In their hands a 2 hour infusion of 120mM K^+ increased 5-HT output by 410%, and omission of Ca^{++} from the aCSF for 2 hours decreased 5-HT levels in dialysate by 58%. Therefore based on the present and previously published findings it is reasonable to assume that the 5-HT measured in dialysate was neuronally derived and subsequent studies could be initiated to look at neuronal function.

Pharmacological Characterisation of the Receptor

The terminal 5-HT autoreceptor has been extensively examined and found to be of the 5-HT_{1B} subtype in the SCN (Martin and Marsden 1986; O'Connor and Kruk 1992) and other brain regions (Martin and Sanders Bush 1982; Engel et al. 1986; Hjorth and Tao 1991). The hypothalamus, but not the SCN (Roca et al. 1993), is well endowed with 5-HT_{1A} and 5-HT_{2C} receptors (Pazos and Palacios 1985), but these are considered to be post-synaptic receptors.

Infusion of increasing concentrations of RU24969 (0.1-10μM) led to a dose-dependent decrease in dialysate 5-HT levels. This dose-dependent effect has not previously been reported. RU24969 (1-5μM), when infused via the dialysis probe, has been shown to increase 5-HT output (Auerbach et al. 1991; Hjorth and Tao 1991) in the absence of an uptake inhibitor. However, when 1μM citalopram, an uptake inhibitor, was included RU24969 (1μM) decreased 5-HT output. This indicates that RU24969 itself can act as an uptake inhibitor, but in the data presented here an uptake inhibitor was already present in the aCSF, and drug solutions, throughout the experiments it can be assumed that RU24969 acted solely as a 5-HT_{1B} agonist. The IC_{50} of RU24969 on [^{3}H]5-HT release *in vitro* is 35nM (Middlemiss 1985), therefore the concentrations of RU24969 used in this study would be sufficient to decrease 5-HT output. The concetrations of RU24969 in the dialysate were 0.1-10μM. Previous, unpublished in vitro studies in the Boots laboratory have shown that approximately 10% of drug leaves the probe. Thus during a 15 min application the amount of RU24969 adminsitered to the anterior hypothalamus can be estimated as being 0.01-1μM. This clearly indicates that the effects of RU246969 are at pharmacologically relevant concentrations.

The inhibitory effects of RU24969 on 5-HT output in the frontal cortex and ventral hippocampus have been demonstrated before *in vivo* (Sleight et al. 1989; Hjorth and Tao 1991; Martin et al. 1992), although Martin and colleagues were the only other workers to show a dose-response relationship in the ventral hippocampus; 0.1μM RU24969 did not significantly decrease 5-HT output whereas 1μM RU24969 decreased 5-HT levels by 47%. This compares with my values of a 49% decrease after 0.1μM RU24969, 56% after 1μM RU24969 and 65% after 10μM RU24969.

To confirm that the output of 5-HT measured in the dialysate was controlled by a 5-HT_{1B} receptor, the selective 5-HT_{1A} agonist 8-OH-DPAT (1μM) was infused. 8-OH-DPAT was infused also because RU24969 has only slightly greater affinity for the 5-HT_{1B} receptor than the 5-HT_{1A} autoreceptor (pK_i 5-HT_{1A} 8.06nM and pK_i 5-HT_{1B} 8.23nM van Wijngaarten et al. 1990). Since 8-OH-DPAT infusion was without effect

on the levels of 5-HT in the dialysate it can be concluded that 5-HT_{1A} receptors do not affect the output of 5-HT measured in this system and that any effect of RU24969 must therefore be due to an action at a 5-HT_{1B} receptor.

Pre-infusion of methiothepin (10μM) blocked the effects of RU24969 (5μM) as described previously (O'Connor and Kruk 1991; Martin et al. 1992). The pre-infusion method was chosen because it gives the antagonist a chance to diffuse throughout the area and bind to 5-HT_{1B} receptors. Infusion of methiothepin alone (10μM and 1μM) increased the overflow of 5-HT. It has been reported previously that methiothepin (1-10μM) elicited a concentration-dependent enhancement of both basal and K^+-stimulated endogenous 5-HT and [^{3}H]5-HT release in hypothalamic slices *in vitro* (Pettibone and Pflueger 1984). O'Connor and Kruk (1991) have also reported an increase in 5-HT levels of 83% after administration of methiothepin (0.1-1μM) in slices containing the SCN. Methiothepin (10mg/kg i.p.) has been shown to increase 5-HT output *in vivo* by up to 57% (Baumann and Waldmeier 1984, Martin and Marsden 1986). Methiothepin is a 5-HT receptor antagonist which shows some selectivity for 5-HT_1 receptors (Hibert and Middlemiss 1986), however it is not entirely selective for 5-HT receptors. Methiothepin is also an antagonist at α_2-adrenoceptors which are known to be present on 5-HT terminals and to have an inhibitory effect on 5-HT release *in vivo* (Hjorth and Tao 1992; Tao and Hjorth 1992) which is tonic (Mongeau et al. 1993). The increased 5-HT output after methiothepin was concentration dependent; the increase after 1μM was 15% and after 10μM 142%. Thus the increased output of 5-HT seen may be attributable partly to inhibition of the autoinhibitory tone of both the 5-HT_{1B} autoreceptor and the α_2 heteroreceptor. However, since methiothepin blocked the effects of RU24969, it is reasonable to conclude that in view of the data discussed above, RU24969 exerts its effects on 5-HT release in the rat via the 5-HT autoreceptor.

Thus the receptor controlling 5-HT release in the anterior hypothalamus can be pharmacologically characterised as a 5-HT_{1B} receptor.

Basal 5-HT Levels at mid light and end light

The dialysate concentrations of 5-HT at mid light was significantly higher than those measured at end light. Experiments *in vivo* using voltammetry or microdialysis probes implanted into the SCN or ventromedial hypothalamus of rats and hamsters have shown that 5-HT and 5-HIAA levels begin to rise at the end of the light phase and peak during the dark phase (Faradji et al.1982; Martin and Marsden 1985; Glass et al. 1992). In the hippocampus, however, 5-HT levels were slightly higher during mid light (20pmoles/60µl) than at end light (15pmoles/60µl) (Kalen et al. 1989). In my experiments significantly more 5-HT was detected at mid light (38fmoles/15µl) than end light (28fmoles/15µl). There are two possible explanations for the differences between my results and those of other workers. Firstly in the experiments of Martin and Marsden (1985), and Glass and colleagues (1992), hamsters and rats were freely moving, so that behaviour, arousal and other external factors might affect the endogenous pattern of 5-HT release, as discussed previously. Whilst outside the scope of the present discussion, these observations question whether the 24 hour rhythm in 5-HT release is indeed an endogenous circadian rhythm. However, very recently Cagampang and Inouye (1994) have presented evidence showing that in the SCN, tissue 5-HT levels exhibit an endogenous 24 hour rhythm. In my experiments rats were anaesthetised and therefore 5-HT levels were not influenced by movement and arousal. Secondly, most of the authors, with the exception of Martin and Marsden (1985) used 5-HIAA as a measure of 5-HT release and there is currently debate as to whether 5-HIAA levels are a true reflection of 5-HT release (Auerbach et al. 1989; Crespi et al. 1990).

Since these experiments were performed in anaesthetised rats, they indicate that the difference in 5-HT release measured at mid light and end light are an inate phenomenon not related to activity or behavioiural state.

Effect of RU24969 at mid light and end light

The effects of a 15 min infusion of RU24969 were significantly different at the

two time points investigated. RU24969 administration caused a quantitatively greater inhibiton of 5-HT output at end light compared to mid light.

The greater effect of infusion of 5μM RU24969 at end light compared to mid light could be due either to a difference in the amount of 5-HT released and therefore receptor occupation, or the sensitivity/number of autoreceptors at the two time points, or to a variation in the second messenger level/activity. A significant variation in 5-HT_{1B} binding has been demonstrated over 24 hours, but only in the cortex (Akiyoshi et al. 1989). The number of sites varied significantly whilst the affinity did not change. The number of binding sites at mid light was significantly higher than at end light which does not correlate with my findings. The differences could be because the technique measured both presynaptic and post-synaptic receptors or the cortical rhythm is different from that in the hypothalamus. mRNA levels for the 5-HT_{1B} receptor in the SCN have been found not to vary over 24 hours (Roca et al. 1993), when measured at four equally spaced time points in the light-dark cycle. However since this is a measure of post-synaptic receptors it does not preclude a rhythm in presynaptic receptors. In the anterior hypothalamus more receptors could be present at end light or the affinity of these receptors could be greatly increased compared to mid light. Another explanation, as extensively discussed in chapter 3, is that there could also be circadian rhythms in receptor-effector coupling, adenylate cyclase activity or the intracellular level of cAMP due to a circadian rhythm in phosphodiesterase as described by Prosser and Gillette (1991) and Perez and colleagues (1991).

Chronic Antidepressant Treatment

Basal 5-HT levels at mid light and end light in treated animals

Rats were treated chronically with two different types of antidepressant; desipramine is a selective noradrenaline uptake inhibitor (K_i NA 0.61nM, 5-HT 180nM and DA 11000nM; Bolden-Watson and Richelson 1993) and paroxetine a selective 5-HT uptake inhibitor (K_i NA 33nM, 5-HT 0.73nM and DA 1700nM; Bolden-Watson and Richelson 1993). Rats were treated for 21 days since the clinical effects of

antidepressant drugs are usually not seen until about 2-3 weeks after initial administration.

Significantly more basal 5-HT was detected in dialysate samples taken from rats treated with desipramine when measured at end light. At mid light, no differences in basal dialysate 5-HT level were detected between saline- and drug-treated rats. This was also the case at end light in those animals treated with paroxetine.

The increase in basal 5-HT levels seen after chronic desipramine treatment at end light may by caused by two, not mutually exclusive, factors. The increase could represent a phase-dependent down-regulation of the terminal α_2-heteroreceptor (Tao and Hjorth 1992) or the cell body α_1-heteroreceptor (Baraban and Aghajanian 1980; Clement et al. 1992a) located on 5-HT neurons. The down-regulation of α_1-heteroreceptors after chronic desipramine treatment may be due to a down-regulation of α_2-autoreceptors, located on NA terminals projecting to the DR from the LC. Frontal cortical α_{1B} binding is decreased after chronic treatment with imipramine, although α_{1A} binding in the frontal cortex and α_{1A} and α_{1B} binding in the hippocampus were unaffected (Hayakawa et al. 1992). However there is as much evidence for an up-regulation (Maj et al. 1985) or no change in α_1-adrenoceptors (Li et al. 1988). The subtype of the α_1-heteroreceptor in the RN has not been determined, nor has its function been assessed after chronic antidepressant treatment. A change in the function of these adrenoheteroreceptors may affect the cell body biophase concentration of 5-HT and thereby in turn down-regulate 5-HT_{1A} autoreceptors. This may then in turn affect firing rate and thus the terminal release of 5-HT, as outlined in the introduction. However, since α_1-heteroreceptors exert a tonic excitatory effect on DR neuronal firing, their down-regulation would decrease neuronal firing, decreasing dendritic 5-HT release and stimualtion of 5-HT_{1A} autoreceptors. Down-regulation of presynaptic α_2-heteroreceptors after chronic desipramine treatment has also been reported (Gonzalez et al. 1992), although although others claim that there is no change in function (Campbell et al. 1992). The effect of prolonged treatment with 5-HT uptake inhibitors on α_2-heteroreceptor function has not been assessed. Singh and Redfern

(1994b) have shown that there is no difference in cortical α_2-heteroreceptor sensitivity through the light-dark cycle in *in vitro*, but the basic agonsit and antagonist studies here were performed during the earlier parts of the light phase. It is unknown whether the α_2-heteroreceptors display a circadian rhythm in the anterior hypothalamus and therfore, no firm conclusions about the cause of the effect of desipramine on basal 5-HT can be drawn.

Effect of RU24969 at mid light and end light in antidepressant-treated animals

The results at mid light will be considered first because it is at this time point that most other assessing neurotransmitter receptor binding and function are carrried out.

When the effect of RU24969 (5μM) was re-assessed after chronic antidepressant treatment at mid light, there was no significant difference in effect between antidepressant- or saline-treated animals. In general neither desipramine nor paroxetine significantly altered the maximal response of the autoreceptor to RU24969 but there was a significant increase in 5-HT output at the end of the experiment. These results are in agreement with the only comparable published study, Sleight and co-workers (1989), if it is assumed that these workers carried out their experiments during the middle of the light phase. These workers found that chronic treatment with either amitriptyline or MDL72394 (a putative MAOI) had no effect on the response to RU24969 (10mg/kg i.p.) in the frontal cortex of the anaesthetised rat. Other studies *in vivo* studies using a different technique have indirectly demonstrated autoreceptor down-regulation in the hippocampus after chronic fluoxetine or citalopram but not clorgyline treatment (Chaput et al. 1986; Blier et al. 1988). In alll these studies rats were chloral hydrate anaesthetised.

Other studies looking at the function of the 5-HT autoreceptor after chronic antidepressant treatment were carried out *in vitro*. However the results were equivocal, reporting both a decrease in sensitivity after treatment with MAOI or 5-HT and 5-HT/NA uptake inhibitors (Maura and Raiteri 1984; Moret and Briley 1990) and a

decrease (Johanning et al. 1992) or no change in the number of 5-HT_{1B} binding sites (Montero *et al.* 1991) after treatment with a 5-HT uptake inhibitor. It is difficult to reconcile these disparate *in vitro* findings with the more physiologically relevant *in vivo* data presented here and by Sleight and co-workers (1988). However the wide variation in both methodology and drug adminsitration may play a significant role.

By contrast, at end light prolonged antidepressant treatment significantly attenuated the effects of RU24969, desipramine having a greater effect than paroxetine. The down-regulation of 5-HT autoreceptors following chronic desipramine treatment might reflect a desensitisation of terminal α_2-heteroreceptors. This desensitisation could be achieved by an increase in NA around the 5-HT nerve terminal following blockade of NA reuptake. Down-regulation of the α_2-heteroreceptor would decrease the feedback inhibiton of this receptor on 5-HT release. The biophase level of 5-HT could then rise and down-regulate the 5-HT autoreceptor. Treatment with paroxetine would have a more direct affect. Blockade of 5-HT reuptake would directly increase 5-HT levels in the synaptic cleft thus allowing desesitisation of the autoreceptor. Since this is the first observation that antidepressant drugs decrease 5-HT_{1B} function *in vivo* it is pertinent to consider the posssible mechanisms that may be involved. As discussed in chapter 3, it may be due to changes in autoreceptor and α_2-heteroreceptor expression, expression of their G protein, uncoupling of the receptor-effector coupling or changes in intracellular signal transduction pathways.

The reason for the greater effect of desipramine is unclear. Clearly, it could be connected to the finding of increased basal levels of 5-HT at end light only after desipramine treatment. As explained earlier in this section, the down-regulation in the α_2-heteroreceptor would increase the terminal biophase concentration of 5-HT and perhaps expose the terminal autoreceptor to a greater level of 5-HT at end light compared to mid light, thus leading to greater down-regulation at this time point.

An antidepressant-induced change in the circadian rhythm in receptor numbers and tissue 5-HT concnetration has been demonstrated before. As mentioned in the Introduction (chapter 1), the peak binding in the daily rhythm of α-adrenoceptors,

GABA-benzodiazepine, α-MSH, DA and mAChR receptors (Kafka et al. 1981b; O'Donahue et al. 1982; Wirz-Justice et al. 1982). receptors is delayed following chronic antidepressant treatment. Additionally, Ozaki and colleagues (1994) have shown that the circadian rhythm of tissue 5-HT concentration from different brain regions is changed following chronic clorgyline administration. Furthermore the changes in rhythm varied according to the region studied. Thus in the SCN and DRN the peak level was delayed, whilst in the paraventricular nucleus of the hypothalamus, medial preoptic area, vLGN and MRN the rhythm was abolished. Therefore, extrapolation of the findings presented here across the 24 hour period suggest either a flattening of rhythms or an alteration of the time of peak effect. Neither of these possibilities is in disagreement with the results described above.

In conclusion, therefore, my results demonstrate that chronic antidepressant drug adminsitration down-regulates 5-HT_{1B} receptor function in a time of day dependent manner. The exact mechanism whereby the alteration in function is achieved is unclear but it is tempting to speculate that it involves one or more of the processes extensively discussed in chapter 3. Nevertheless, the present data stress the need to examine receptor function throughout the 24 hour cycle to determine whether chronic treatments induce adaptational changes.

Summary

In summary, 5-HT output in the anterior hypothalamus is controlled by a 5-HT_{1B} autoreceptor which appears to display autoinhibitory tone. Terminal 5-HT_{1B} receptor function displays a marked diurnal variation when compared at mid light and end light. Perhaps the most important finding is that chronic treatment with paroxetine or desipramine changed the function of the autoreceptor in a phase-dependent manner. Chronic antidepressant treatment did not affect the receptor at mid light, but significantly down-regulated the 5-HT_{1B} receptor at end light. In fact, chronic antidepressant treatment made the response to RU24969 at mid light and end light equal. This was the case with both antidepressants which target either the 5-HT or NA

system selectively. Down-regulation only occurred at one time point, which provides good evidence for the hypothesis of this thesis that antidepressant treatment would affect 5-HT_{1B} control of 5-HT release only at some time points.

Chapter 5 General Discussion

The aim of this chapter is to synthesise the results from preceeding experimental chapters outlining similarities and differences in results and possible reasons for those anomalies. Additionally I shall discuss how the results tally with the original hypothesis and how the hypothesis might be expanded, as well as indications of future work needed to enlighten "dark corners".

Synthesis of experimental findings

Three different techniques were used to assess autoreceptor control of synthesis and release. However since one technique proved unsuccessful this section will mainly concentrate on bringing together the results from the 5-HTP accumulation and microdialysis studies.

Uptake of [^{3}H]5-HT into hypothalamic slices varied over 24 hours, being considerably higher at end light but not significantly different at the other three time points. It is possible that the degree of uptake may have affected the basal synthesis rate of 5-HT. As outlined in the discussion in chapter 3, the biophase concentration of 5-HT could feed back to affect the rate of synthesis. If 5-HT uptake is higher at end light then it might be expected that the synthesis rate would be higher as a result of less negative feedback. However the synthesis rate was quantitatively highest at mid dark, and significantly higher at mid dark than at end light. Either the rhythm in [^{3}H]5-HT uptake is not a major factor *in vivo* or alternatively the difference could be due to the use of hypothalamic slices and [^{3}H]5-HT. Experiments using *in vivo* microdialysis indicated that 5-HT levels were higher at mid light than end light. Thus in the hypothalamus it might be expected that the synthesis rate would be lower at mid light than at end light. Again these results do not tally with the variation in synthesis rate observed which may be due to anterior hypothalamus vs whole hypothalamus.

The higher uptake of [^{3}H]5-HT at end light could lead to a reduction in the biophase level of 5-HT. This would decrease the competition between 5-HT and

RU24969 for the 5-HT_{1B} binding site. However in the hypothalamus the RU24969-induced decrease in 5-HTPacc did not vary over 24 hours. Therefore the uptake might be higher at this time point because 5-HT release is greater, although the results of the *in vivo* microdialysis studies argue against this.

In dialysate the 5-HT levels was Ca^{++}- and agonist-dependent which is in direct conflict with the results from superfusion studies. The release seen in both studies was, however, K^+-dependent. This indicates that an increased output of 5-HT after elevating the K^+concentration does not necessarily reflect an increased neuronal release.

Administration of RU24969 had different effects on synthesis and release over 24 hours. The results from *in vivo* microdialysis experiments demonstrated a significant difference in the effect of RU24969 at mid light compared to end light. In contrast, RU24969 had the same effect on 5-HT synthesis rate measured through the light-dark cycle. Again this difference might be due to the use of anterior hypothalamus vs whole hypothalamus.

Basal TrOH activity was not significantly different after chronic antidepressant treatment at end light, however basal 5-HT levels, measured by *in vivo* microdialysis, were higher at end light after chronic desipramine treatment. The difference could be accounted for because different parameters were being measure, the treatment schedules of the rats used were different or perhaps α-adrenoheteroreceptors do not have as great an effect on synthesis as they do on release.

The 5-HT_{1B}-mediated control of synthesis was attenuated by chronic desipramine treatment at mid light and chronic paroxetine treatment at end light. However 5-HT_{1B}-mediated control of release was down-regulated by both antidepressant drugs only at end light. Again the difference might be attributable to variations in the dosing schedule. The timing of administration of the antidepressant drugs could be very important in its effect on receptor populations. Animals used in *in vivo* microdialysis experiments were always injected during the latter half of the light phase, whilst those used for synthesis experiments were randomly injected. There are no studies on the chronopharmacokinetics of desipramine or paroxetine. The fact that

antidepressant-treated rats were treated whilst being phase-shifted may also account for the difference, although published data do not support this contention. The rats came from different suppliers; rats used for dialysis experiments were bought from Olac, whilst those used for synthesis experiments were bred at the University of Bath. Again the differences in down-regulation might be due to the fact that the same parameter was not being measured. Possibly the intracellular pathway for release, involving synapsin, synaptophysin and other cytoskeletal elements might react differently fom intracellular factors affecting synthesis, involving PKA, activator proteins and/or TrOH.

Thus the results taken together indicate that chronic antidepressant treatment affects 5-HT_{1B}-mediated control of synthesis and release over 24 hours in a differential manner.

Hypothesis tested

The initial hyothesis of this thesis was that prolonged treatment with antidepresant drugs alters the 24 hour pattern of control the terminal 5-HT_{1B} autoreceptor exerts on 5-HT synthesis and release.

This prediction was based on elements from several lines of evidence. On one hand, 5-HT has been implicated in the aetiology of depression for many yeras and many clinically effective antidepressant drugs target the central serotoninergic system. Secondly, in at least a subpopulation of depressives, a malfunction of the circadian system has been demonstrated. Additionally, in laboratory animals some antidepressants have been shown to alter circadian rhythms in locomotor activity and in receptor populations. Finally, several factors are known to affect the activity of serotoninergic neurons e.g. autoreceptors and circadian rhythms.

Based on this original hypothesis, a model has been developed to explain how antidepressant drugs interact with the 5-HT system, via 5-HT_{1B} receptors, to modualte circadian rhythms and therby exert therapeutic effects.

Model of interaction between 5-HT, antidepressant drugs and ciradian rhythms

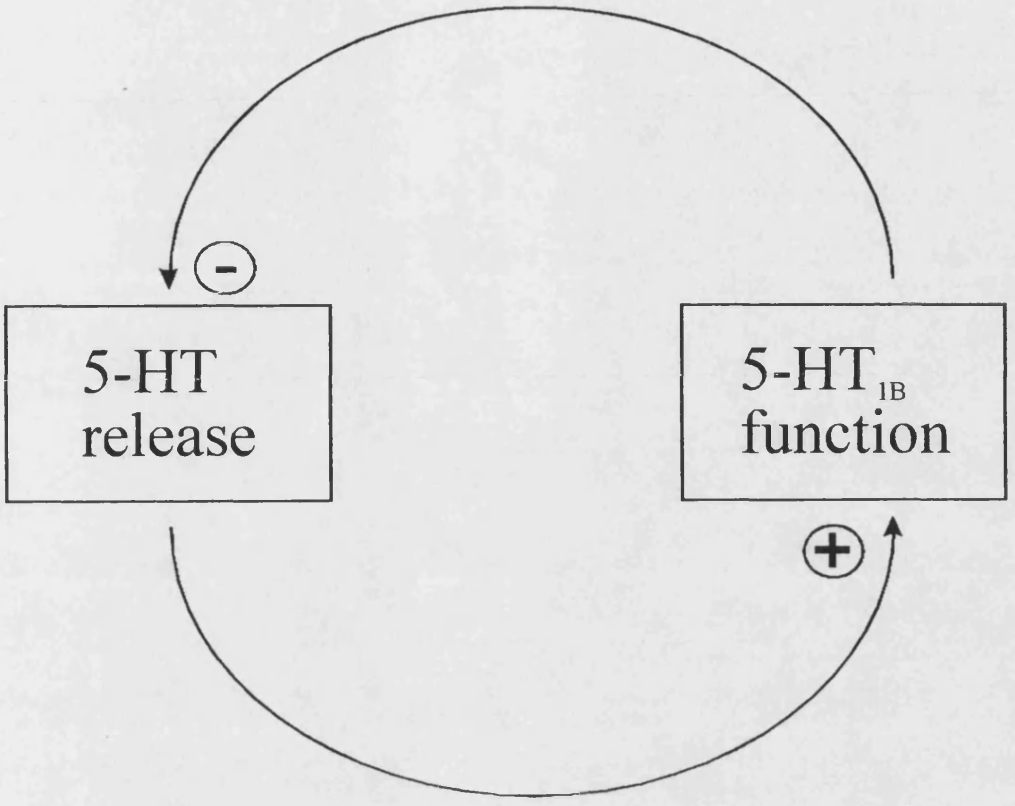
The proposed model suggests that in a certain subset of depressed patients, the regulation of 5-HT_{1B} autoreceptor expression or post-translational processing is abnormal, leading to an overexpression or increased affinity of these autoreceptor. Thus when 5-HT is released and the autoreceptors are stimulated the inhibitory effect of these autoreceptors decreases 5-HT synthesis and release and therefore the amount of 5-HT released per impulse, further increasing the number or sensitivity of the autoreceptors. Such as process clearly leads to a self-reinforcing positive feedback loop. Under normal conditions autoreceptor function and release are correlated; when the extracellular 5-HT level rises, the autoreceptor stimualtion is greater and feedbak inhibition increased. If the autoreceptor function is abnormal, then this relationship breaks down; this is shown diagrammatically in figure 47 and explained in the legend. The breakdown in the relationship affects the biophase concentration of 5-HT over 24 hours and therefore stimualtion of post-synaptic targets, some of which may be central elements in the circadian system.

The model further proposes that antidepressant drugs exert their therapeutic actions in this subset of depressives by altering the function of autoreceptors by up- or down-regualtion, depending on the time of day, to "normalise" the activity of the autoreceptor. This normalisation of the 5-HT input to the biological clock may reset circdian rhythms and thereby help to alleviates depression.

The data presented in this thesis, in conjunction with published work, can be used to validate the model. However, since the model is complex it will be broken down into its constituent parts.

5-HT_{1B} receptors in depression

There is good evidence for an alteration in the function of the 5-HT system in at least a subpopulation of depressives. For example, plasma l-try levels are significantly lower in depressed patients (Cowen et al. 1989; Pietraszek et al. 1991). In depressed patients previously successfully treated with antidepressant drugs, rapid



5-HT release	5-HT_{1B} autoreceptor function
↑	↓
↓	↑

Figure 47. Relationship between 5-HT autoreceptor function and extracellular 5-HT level. Under normal conditions 5-HT release and autoreceptor function are well correlated. As the extracellular concentration of 5-HT increases it feeds back onto the autoreceptor to decrease further 5-HT release. However, if the autoreceptor pattern is altered, by changes in receptor expression, then this feedback mechanism will break down and affect the pattern in 5-HT release.

depletion of tryptophan induces a relapse into a depressed state (Delgado et al. 1990). Their state improves when a normal diet is given. Neuroendocrine challenge tests also provide evidence for a serotonin abnormality in depressives (Lesch et al. 1990; Price et al 1991).

The function of the terminal 5-HT autoreceptor is very important since it controls release and therefore the biophase concentration of 5-HT, it can respond rapidly to changes in extracellular 5-HT levels, adjusting release as necesary.

Although direct evidence of a 5-HT_{1B} malfunction in depression is not found in the literature, the results of several experiments indirectly point in this direction. The increase in post-synaptic 5-HT_{1D} (Lowther et al. 1991), 5-HT_2 (Stanley and Mann 1982) and 5-HT_1 (Cheetham et al. 1990) receptor density demonstrated in depression could indicate a functional up-reguation of these receptors in response to the decrease in 5-HT release due to a supersensitive terminal autoreceptor. Similar changes in post-synaptic receptors are seen after neurochemical lesion of the 5-HT system (Underwood et al. 1992).

5-HT_{1B} autoreceptor function over 24 hours

The data in this thesis have demonstrated that, in the anterior hypothalamus, hippocampus, frontal cortex and striatum, 5-HT_{1B} receptor function varies over 24 hours. This is in contrast to the findings of Singh and Redfern (1994a) who have shown that *in vitro* 5-HT_{1B} autoreceptors in the frontal cortex do not display any rhythm.

However, several other workers have presented evidence for rhythms in 5-HT_{1B} receptors. Martin and colleagues (1987) have demonstratd a circadian rhythm in post-synaptic 5-HT_{1B} receptors in the mouse using a behavioural model. These workers found that the receptor was more sensitive during the dark phase. Prosser and co-workers (1993) have shown that 5-HT_{1B} binding is higher during the middle of the dark phase compared to the middle of the light phase in the SCN. In the cerebral cortex 5-HT_{1B} binding is higher during the light phase than the dark phase (Akiyoshi et al. 1989). In situ hybridisation, though, has detected no change in mRNA for 5-HT_{1B}

receptors in the SCN (Roca et al. 1993); however since this is a measure of receptor expression in the cell body it will not measure presyanptic 5-HT_{1B} receptors. Thus the work in this thesis and evidence from published studies demonstrates a 24 hour variation in 5-HT_{1B} receptors.

The data presented in this thesis do not prove a circadian rhythm in 5-HT_{1B} function, but rather illustrate that 5-HT_{1B} function varies over 24 hours. Further studies would be required to assess whether the observed variation is truly circadian.

In normal subjects 5-HT_{1B} function correlates with 5-HT release. The results of the *in vivo* microdialysis studies demonstrate that autoreceptor function is higher when release is decreased. If the relationship between release and autoreceptor is changed, either by abnormal expression or by post-translational processing changes it might alter the circadian fluctuation in the autoreceptor, thus breaking down the feedback mechanism and changing the biophase concentration of 5-HT over 24 hours.

Role of 5-HT in the control of circadian rhythms

The circadian system can be thought of as consisting of three parts; an input to the pacemaker relaying information about the external environment, the pacemaker itself and outputs from the pacemaker to the integration/effector areas.

The main mammalian pacemaker is considered to be the SCN, an area of the anterior hypothalamus (Stephan and Zucker 1972; Rusak and Groos 1982; Ralph et al. 1990). The SCN is a bilateral nucleus located at the base of the third ventricle above the optic chiasm which SCN receives external information via two pathways, the retinohypothalamic and geniculohypothalamic tracts, RHT and GHT respectively. The RHT is a direct pathway from the retina to the hypothalamus and is considered to be necessary for entrainment (Johnson et al. 1988), whilst the GHT is an indirect pathway passing from the retina to an area of the ventral lateral geniculate nucleus (vLGN) called the intergeniculate leaflet (IGL). Having synapsed in the IGL the pathway passes to the SCN; it is considered that part of the GHT is a collateral of the RHT (for a comprehensive review of the visual relays to the SCN see Card and Moore 1991). The

GHT is thought to have a modulatory role, not being necessary for entrainment but rather relaying information about light intensities (Pickard et al. 1987). Both retinal afferents synapse in the ventral lateral portion of the SCN.

The SCN, which contains high levels of 5-HT (Saavedra et al. 1974) receives their most robust innervation from the RN. The DR projects to the SCN via the dorsal raphe periventricular tract and a projection from the DR that also innervates the IGL. Indeed collaterals of the same DR neuron can innervate both the SCN and IGL. The MR pathway passes in the median forebrain bundle to the SCN (Azmitia and Segal 1978).

Serotonin has been implicated in the control of circadian rhythms for many years, although only recently have data indicating its true importance started to emerge. The results stem from three different types of experiment; depleting or increasing 5-HT throughout the brain *in vivo*, adminstration of 5-HT agonists *in vivo* and *in vitro*.

Whole animal studies

If the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) is injected intraventricularly then it selectively destroys 5-HT neurons in the dorsal raphe (DR) and decreases 5-HT in DR projection areas (Dewar et al. 1992). It is important to note that the median raphe (MR) does not appear to be affected, so 5-HT will still be present in those areas that receive MR projection although the actual amount of 5-HT in each region will depend on the degree of innervation by the MR. Only two studies have thoroughly investigated the effect of 5,7-DHT on the circadian system by measuring the circadian pattern of wheel-running activity in hamsters. When entrained to a light-dark cycle, hamsters lesioned with 5,7-DHT began wheel running earlier and stopped wheel-running later than sham-operated hamsters (Smale et al. 1990; Morin and Blanchard 1991). When the hamsters were moved to constant light the total "day" length (circadian period, tau) of 5,7-DHT-lesioned hamsters was significantly longer compared to sham-operated hamsters. In constant light lesioned hamsters tended to

become arrhythmic i.e. the activity was not clearly defined into bouts. When these hamsters were then moved to constant darkness lesioned hamsters took longer to resume a normal circadian pattern of activity than sham-operated control hamsters (Morin and Blanchard 1991). Re-entrainment to an 8 hour advance in the light-dark cycle was not smooth in 5,7-DHT-lesioned hamsters compared to sham-operated controls (Smale et al. 1990). When the hamsters were put into constant darkness the circadian period was more variable in lesioned hamsters, showing large increases and decreases. The re-establishment of a stable free-running rhythm in sham-operated hamsters was smooth and normal, however lesioned hamsters did not establish free-running rhythms smoothly and tended to lapse into a desynchronised state (Smale et al. 1990).

One measure of the circadian system is the phase response curve (PRC). The circadian wheel-running activity of hamsters kept in constant dark can be altered by delivering light pulses at specific times. Light pulses delivered early in the subjective night delay the hamster's activity, whilst pulses delivered late in the subjective night advance the animal's wheel running activity; during the subjective day there is a dead zone where no change in activity is recorded. The phase shift i.e. the advance or delay in activity, in response to light pulses delivered at specific circadian times is plotted as a PRC. In 5,7-DHT lesioned hamsters the phase delays in response to light pulses delivered at CT13-15 were significantly greater than sham operated control hamsters. The whole PRC was shifted by about one hour, indicating that lesioned animals were sensitive to light for a prolonged time span (Morin and Blanchard 1991).

These studies show that DR 5-HT neurons are important for maintaining endogenous pacemaker function and entrainment. The role of the MR has not been fully investigated.

Penev and colleagues (1993) have shown that the onset and offset of wheel-running activity was advanced in entrained hamsters after reserpine treatment. It was also noted that in constant darkness there was an increase in free-running period. Moreover the PRC was altered follwing reserpine pre-treatment. The phase advance in

locomotor activity after light pulses at CT19 was significantly greater than in control animals.

Increasing the level of 5-HT is achieved by chronic treatment with the MAO-A inhibitor, clorgyline. It should be noted that in hamsters 90% of the MAO is the A isoform so not only 5-HT but also NA and DA will be increased. When clorgyline was administered to hamsters kept in constant darkness it increased their circadian period and delayed their activity onset and offset (Duncan et al. 1988). If hamsters were clorgyline-treated, following entrainment to a light-dark cycle, their circadian period was also increased (Duncan et al. 1988). The pattern of activity in the dark phase was different in clorgyline-treated hamsters; the nocturnal activity of control hamsters was concentrated into the first portion of the dark phase, whilst clorgyline-treated animals were active throughout the dark phase (Duncan et al. 1988). The PRC of hamsters treated with clorgyline was significantly different from saline-treated hamsters. The maximum phase delay occurred 2 hours later and phase advances were not as great in clorgyline-treated animals (Duncan et al. 1988). Tamarkin and colleagues (1983) noted that under a light-dark cycle, clorgyline-treated hamsters had delayed activity onsets and offsets. The activity onsets were advanced throughout the course of the experiment but the hamsters never regained their pre-treatment activity onset times. The delay in activity onset brought about by clorgyline treatment was dose-dependent. If the beginning of the dark period was advanced by 2 hours, saline-treated hamsters entrained within 7 days; clorgyline-treated hamsters gradually advanced their activity onset but never to the same extent as saline-treated hamsters and never achieved a new entrained rhythm (Tamarkin et al. 1983). When hamsters were entrained to a light-dark cycle, then treated with clorgyline or saline and subsequently subjected to constant darkness the clorgyline-treated hamsters had further delayed activity onsets and offsets (Tamarkin et al. 1983).

The results of the above studies indicate that although 5-HT is not necessary for the generation of circadian rhythm it appears to play a role in modulating the effect of light on the circadian pacemaker, although the locus of action is not clear. One

further experiemnt lends weight to the involvement of 5-HT in circadian rhythms. If RU24969 is infused directly into the SCN of entrained rats, their locomotor activity becomes arrhythmic (Martin and Marsden 1986b). This result also suggests that inhibition of 5-HT release abolishes entrainment in rats. The findings of Martin and Marsden (1986b) may help to explain the link between decreased 5-HT release, disturbed circadian rhythm and depression. Figure 48 shows the levels at which the serotoninergic system could affect the circadian system. In addition the DR has recently been shown to receive a direct retinal projection in rats (Shen and Semba 1994).

In vitro studies

The neuronal firing rate of SCN cells can be measured *in vitro* in slices of hypothalamus containing the SCN. In this system the cells display a circadian rhythm in their firing rate, as they do *in vivo*, and the firing rate remains stable for several days *in vitro*. If 5-HT is administered either as a microdrop onto the ventrolateral portion of the SCN (Medanic and Gillette 1992) or as a bath infusion (Shibata et al. 1992; Prosser et al. 1993) it can shift the circadian rhythm in SCN neuronal firing for up to 3 days after first administration. Applications during the light phase advance the neuronal firing rhythm (Medanic and Gillette 1992; Shibata et al. 1992; Prosser et al. 1993) and the effect is mimicked by 8-OH-DPAT and 5-carboxamidotryptamine (5-CT) (Medanic and Gillette 1992) a selective 5-HT_{1A} agonist and a 5-$HT_{1A/B}$ agonist respectively (van Winjgaarten et al. 1990). Quipazine (which has affinity for virtually every 5-HT receptor, the 5-HT transporter and a variety of other neurotransmitter receptors, van Wijngaarten et al. 1990) has also been shown to dose-dependently phase advance the firing rhythm (Prosser et al. 1993), but unlike the above drugs it caused phase delays when administered during the dark phase. Shibata and colleagues (1992) showed small but non-significant phase delays in firing rate during the dark phase. The effects of quipazine during the light phase were antagonised by NAN190 (which also blocks α_1-adrenoceptors, van Wijngaarten et al. 1990), but interestingly neither of these antagonists blocked quipazine's effects during the dark phase. The field potentials after optic nerve stimulation *in vitro* can be measured in hypothalamic slice preparation

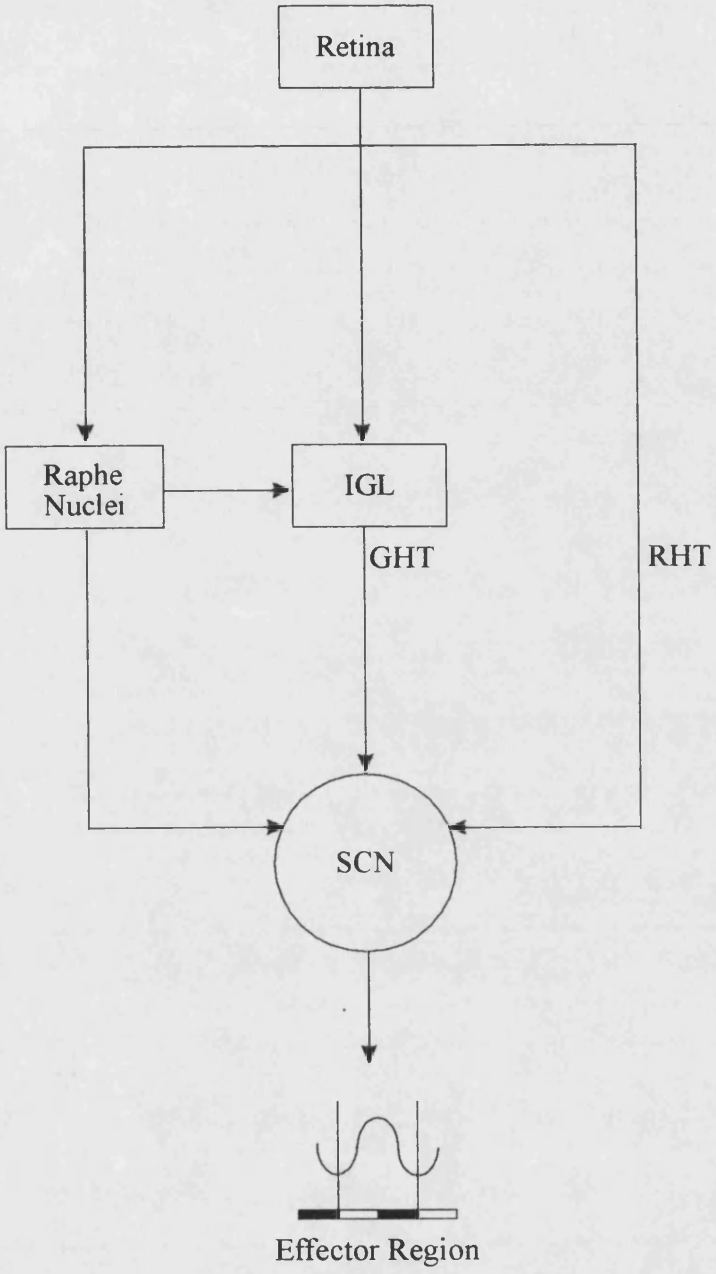


Figure 48. Schematic diagram of the circadian system indicating afferent pathways to the central circadian pacemaker, the suprachiasmatic nucleus (SCN). Information about the external environment reaches the SCN via two pathways from the retina; a direct projection from the retina to the SCN, the retinohypothalamic tract (RHT) and an indirect pathway that passes from the retina via the intergeniculate leaflet (IGL) to the SCN. In addtion, the SCN receives a robust projection from the dorsal and median raphe nuclei (DR and MR respectively). The DR itself receives an afferent from the retinal and can innervate the IGL, some of the axons being collaterals of those fibre passing to the SCN.

incorporating the SCN. These field potentials have been shown to be inhibited by 5-HT and 8-OH-DPAT in a dose-dependent manner (Rea et al. 1993). This shows that 5-HT can directly modulate the RHT input to the SCN. The spontaneous and light-induced activity in firing rate of IGL cells was blocked by administration of 8-OH-DPAT in a dose-dependent manner (Ying et al 1993). The effect of 8-OH-DPAT was antagonised by the non-specific 5-HT antagonist metergoline but not a 5-HT_{1A} antagonist.

Specific in vivo studies

Two *in vivo* studies have further implicated 5-HT in the mediation of circadian rhythms. Administration of 8-OH-DPAT during the light phase advances the wheel running activity of rats (Tominaga et al. 1992). In addition 8-OH-DPAT administration significantly accelerated the rate of re-entrainment to an 8 hour advance in the light-dark cycle (Tominaga et al. 1992). The light-induced phase shifts in hamster's activity described earlier (PRC) can be blocked by 8-OH-DPAT; both advances and delays being inhibited. In the SCN and vLGN post-synaptic neurons are more responsive to 5-HT when it is administered during the dark phase (Mason 1986). Interestingly when the SCN was lesioned the rhythm in responsiveness of vLGN neurons was abolished; however depleting 5-HT throughout the brain did not modify the circadian variation in sensitivity of SCN neurons (Mason 1986). Therefore these studies demonstrate that post-synaptic receptors profoundly alter circadian rhythms in neuronal firing or behaviour and hence potentially pacemaker activity. Extrapolation of my findings in the hypothalamus to the SCN in particular, suggest that if the rhythm in 5-HT release is modified as a result of manipualtion of 5-HT_{1B} autoreceptor function, then circadian rhythms in SCN neuronal firing and behaviour might be affected.

The above *in vivo* and *in vitro* experimental data also imply that the effects of 5-HT on circadian rhythms are mediated by a post-synaptic 5-HT_{1A} receptor. Prosser and colleagues (1993) have demonstrated 5-HT_{1A} binding sites in the SCN, though the number during the light phase and dark phase were reported to be the same. However Roca and co-workers (1993) could not detect any mRNA for 5-HT_{1A} receptors in the SCN. This is interesting because it implies there are no post-synaptic 5-HT_{1A} receptors

on other cell bodies in the SCN, which is at odds with the findings of Prosser and colleagues. One possibility is that the terminal autoreceptor in the SCN is of the 5-HT_{1A} subtype; The evidence presented in this thesis, together with that of Martin and Marsden (1986a) and O'Connor and Kruk (1992) argues against this. The possibility that the 5-HT_{1A} receptor is located presynaptically on retinal afferent terminals themselves cannot be ruled out. A recently cloned 5-HT receptor, termed 5-HT_7, might explain the discrepancy in the results. The new human receptor, cloned and characterised in 1993 by Bard and colleagues is positively coupled to adenylate cyclase by a G protein. This fact could explain the findings of Prosser and Gillette (1989) that cAMP and its analogues can phase shift the circadian rhythm of SCN neuronal firing *in vitro*. The new receptor, transfected in monkey kidney cells and shows high affinity for 5-HT (K_i 8.1nM) in binding studies, additionally it is very sensitive to 5-CT (K_i 0.93nM) and metergoline (K_i 6.4nM); however the receptor shows low affinity for 8-OH-DPAT (K_i 466nM) (Bard et al. 1993). The new receptor has also been isolated from rat hypothalamic tissue (Lovenberg et al. 1993). It too shows highest affinity for 5-CT (IC_{50} 0.83nM), and relatively high affinity for methiothepin (IC_{50} 1.3nM), 5-HT (IC_{50} 9nM), 8-OH-DPAT (IC_{50} 98nM) and metergoline (IC_{50} 30nM). The receptor is found in the thalamus, hippocampus, cortex and various hypothalamic nuclei. However detection of mRNA for the 5-HT_7 receptor was not consistent in the SCN, being at the lower detection limit of the technique. Using the circadian rhythm in SCN neuronal firing *in vitro*, Lovenberg and workers (1993) found that the 8-OH-DPAT-induced phase-shift in neuronal firing was unaffected by a 5-HT_{1A} antagonist, but blocked by an antagonist which displays higher affinity for the 5-HT_7 receptor than the 5-HT_{1A} receptor. This evidence implies that the effects of 5-HT on the circadian rhythm in neuronal firing are mediated by a 5-HT_7 receptor.

The SCN can be divided into two portions on the basis of distinct neurotransmitter distribution. The dorsomedial section contains high levels of vasopression whilst the ventrolateral portion has one of the highest concentrations of vasoactive intestinal polypeptide (VIP) in the brain (Beinfield et al. 1984). Retinal

afferents, from both the RHT and GHT, have been shown to terminate on VIP neurons in the ventrolateral SCN (Hisano et al. 1988; Ibata et al. 1988). The serotoninergic projection to the SCN terminates in the same area as retinal afferents and in addition VIP neurons are a prime target for 5-HT in the SCN (Bosler and Beaudet 1985). There are indications that NPY can alter 5-HT synthesis in the hypothalamus (Vallejo et al. 1987) and 5-HT terminals have been demonstrated to possess NPY heteroreceptors (Schlicker *et al.* 1991); NPY being the neurotransmitter of the GHT (Albers and Ferris 1984). Thus 5-HT can modulate RHT neurotransmission, has close links with the GHT in the SCN and affects neurons in the IGL, placing it in an important position in one of the key areas of the circadian system. The function of the autoreceptor controlling 5-HT release is therefore very important since it will mediate the biophase concentration of 5-HT and thus control stimulation of post-synaptic neuronal elements, possibly involved in the circadian system.

Circadian rhythms and depression

The experimental evidence for circdian rhythm abnormalities in depression is reviewed in the Introduction (chapter 1). This section aims to provide a succinct review of the key findings.

Many studies have shown specific abnormalities in the circadian rhythms of different parameters in depressed patients. The circadian rhythm in plasma prolactin, corticotropin, cortisol, GH, thyrotropin and melatoninn is altered in depressives but there is no consensus as to the nautre of the change. Additionally body temperature, heart rate, sleep timing and architecture are all altered in depression, although again the precise changes vary significantly (Hallonquist et al. 1986; van cauter and Turek 1986; Goetze and Tolle 1987; Daimon et al. 1992). The circadian period of core body temperaturee tended to be longer in depressives (Daimon et al. 1992). the early morning increase in ACTH and nocturnal rise of prolactin and GH all occur earlier in depressed patients (van Cauter and Turek 1986). Interestingly the amplitude in the circadian rhythm of plasma l-try is dampened in depressed patients (Candito et al. 1992).

This evidence demonstrates that circadian rhythms malfunction in depression but they do not indicate the locus of the abnormality.

Antidepressant drugs and 5-HT_{1B} receptors

Several studies have investigated the effect of chronic antidepressant drugs on the terminal 5-HT autoreceptor. The results, though, are equivocal, *in vitro* studies have demonstrated down-regualtion of the autoreceptor (Maura and Raiteri 1984; Moret and Briley 1990; Blier and Bouchard 1994) but not always (Blier and Bouchard 1994), on the other hand chronic antidepressant treatment has been shown either to decrease (Johanning et al. 1992) or not to effect the number of 5-HT_{1B} binding sites (Montero et al. 1991). Studies performed *in vivo* also provide conflicting evidence; Sleight and colleagues (1988) have provided proof that ther is no autoreceptor down-regulation after adminsitraitron of a reuptake inhibitor or a MOAI, conversely Blier and co-workers (1988) and Chaput et al. (1986) have demonstrated down-regulation after treatment with SSRIs but not a MAOI.

The experimental findings of this thesis agree with some of the *in vitro* findings. At a comparable time point (mid light) in the anterior hypothalamus the autoreceptor was unaffected by antidepresssant treatment. However in the whole hypothalamus and frontal cortex down-reglation was observed. Interestingly, at the same time point, up-regulation of 5-HT_{1B} receptors was witnessed in the hippocampus and striatum.

Antidepressant drugs and 24 hour variation in 5-HT_{1B} receptor function

This thesis has demonstrated a time of day dependent up- and down-regulation in 5-HT_{1B} receptor function following prolonged antidepressant adminsitration. This up- and down-regulation alters the 24 hour pattern of control the 5-HT_{1B} receptor exerts on 5-HT synthesis and release, however the pattern in each brain region studied was affected differently.

It is not possible to compare these data with published results because this work is novel. The ability of antidepressant treatment to alter the 24 hour variation in

binding of other receptor populations has been dewmonstrated before (Kafka et al. 1981b; O'Donahue et al. 1982; Wirz-Justice et al. 1983). However the data presented her are the first demonstration of chronic antidepressant-induced alteration in 5-HT_{1B} control of synthesis and release over 24 hours.

Interaction between 5-HT, antidepressant drugs and circadain rhythms

Previous sections of this discussion have suggested how 5-HT_{1B} autoreceptors may be defective in depression, the involvement of the 5-HT system in depression and circadian rhythms and finally how antidepressant drugs alter 5-HT autoreceptor function and change the relationship between 5-HT release and autoreceptor function.

To bring all these ideas together, the model developed proposes that antidepressant drugs when chronically administered, are effective in alleviating depression with a circadian element because they alter a malfunction in 5-HT_{1B} autoreceptor control of synthesis and release by up- and down-regulating the autoreceptor in a time of day dependent fashion. This antidepressant-induced change in receptor function therefore alters the 5-HT input to the biological clock, in effect resetting the clock. This hypothesis is depicted in figure 49.

One interesting question that arises is that, if antidepressant drugs owe their clinical efficacy to their ability to reset circadian rhythms, why do all depressed patients show unequivocal signs of circadian rhythm abnormalities? Depressive illness in which altered circadian rhythms are implicated might represent a subpopulation of patients in whom there is a malfunction of the 5-HT system in key circadian "control centres", specifically, the IGL and SCN, in addition to its deficiency in other brain regions already mentioned. If less 5-HT were released in the SCN and IGL, because of autoreceptor supersensitivity (presynaptic 5-HT_{1B} receptors have been detected in the LGN; Boschert et al. 1994), the modulatory role of 5-HT in these two key areas could be lost. Chronic antidepressant treatment, by increasing the biophase level of 5-HT and resetting disturbed autoreceptor function could normalise the 5-HT input to these two areas of importance of the biological clock. It is not inconceivable that antidepressant

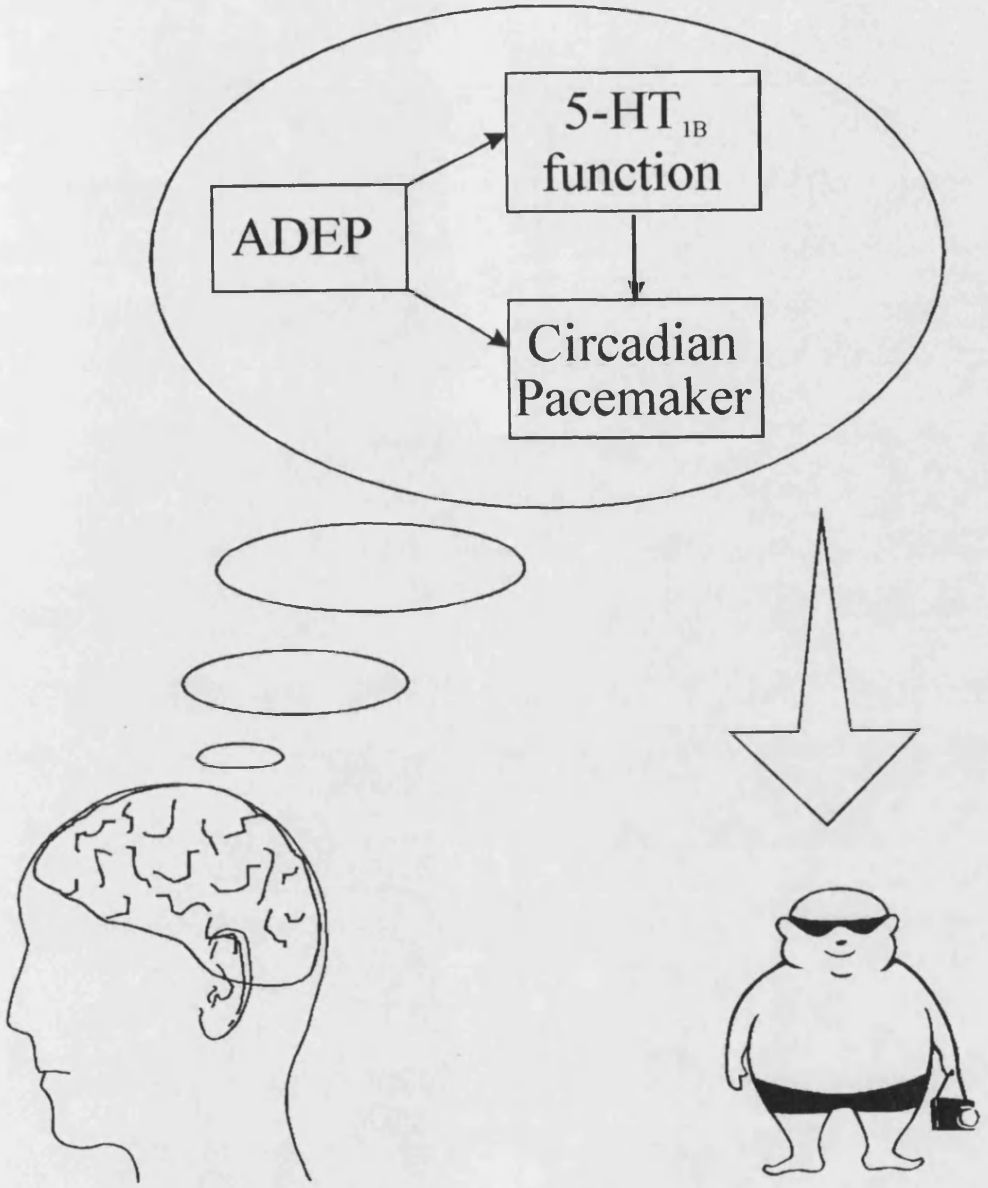


Figure 49. Proposed model for the mechanism by which chronic antidepressant treatment resets circadian rhythms via an action at the 5-HT_{1B} autoreceptor. In depression a supersensitive autoreceptor is expressed which leads to feedback inhibition of 5-HT synthesis and release. This abnormality breaks down the feedbcak relationship between 5-HT release and autoreceptor function altering 5-HT release over 24 hours. The change in the 24 hour pattrn of release affects post-synpatic targets in such areas as the IGL and SCN thus affecting circadian rhythms. Administration of antidepressants (ADEP) up- or down-regulates the 5-HT autoreceptor in a time of day dependnet fashion, which normalises the 5-HT input to the circadian system and resets circdian rhythms thus alleviating depression.

administration increases 5-HT levels only in specific brain regions. The data in this thesis and published work also indicate that 5-HT receptors in different brain regions of the same animal can be differentially affected after prolonged administration of the same antidepressant (Martin et al. 1992b; Blier and Bouchard 1994). The results of this study indicate that antidepressant treatment can reset 5-HT_{1B} control of release and synthesis supporting this theory, although 5-HT function in these two areas was not actually assessed.

Two studies have specifically investigated the effect of chronic antidepressant treatment on responses to 5-HT in the IGL and SCN. In both cases the antidepressant was imipramine which inhibits the reuptake of both 5-HT and noradrenaline. Meijer and Groos (1988) found that chronic imipramine treatment decreased the spontaneous discharge rate of neurons in the SCN and vLGN areas equally during the dark phase and light phase. Harrington and Rusak (1991) also found that the discharge rate of IGL neurons was decreased after chronic imipramine treatment. Both these lines of evidence indicate that chronic antidepressant treatment can alter 5-HT function in key areas of the circadian system which leads to an effect on the endogenous neuronal properties of these areas. The hypothesis could be tested by eliminating presynaptic 5-HT_{1B} autoreceptors by infusion of antisense oligodeoxynucleotides (ODN) into the RN before antidepressant treatment. To verify which raphe input, the DR or the MR, was most important for the mediation of circadian rhythms the ODNs could be infused to either RN and the effect on activity rhythms, IGL and SCN neuronal firing measured.

Conclusion

This thesis has investigates the hypothesis that chronic antidepressant treatment alters 5-HT_{1B} autoreceptor control of synthesis and release in a phase-dependent manner. The results of the work presented in this thesis agree with this hypothesis. 5-HT synthesis and release were influenced by a 5-HT_{1B} receptor and the degree of control the receptro exerted varied signficantly over 24 hours. Chronic antidepressant treatment signficiantly altered the 24 hour pattern of control, causing both up- and

down-regualtion. Moreover the effect of antidepressant treatment is not confined to those antidepressant drugs that only directly affect the 5-HT system; the results with desipramine implicate the noradrenergic system, at least in its ability to influence the 5-HT system. It can therefore be concluded that prolonged administration of antidepressant drugs affects the 24 hour control of synthesis and release.

On the basis of this conclusion I have developed a model to explain how antidepressant drugs alleviate depression with a circadian element. Factors that affect autoreceptor function will influence autoreceptor control of the circadian biophase 5-HT level, in turn affecting 5-HT modulation of converging post-synaptic signals. In those depressive illnesses characterised by a malfunction of the circadian system antidepressant drugs affect the abnormal 5-HT_{1B} autoreceptor control of 5-HT release and synthesis in key areas of the circadian system. This control could be exerted at the input level or within the SCN itself, or both. Thus the model proposed here could accommodate the range of hypotheses of circadian abnormality in the clock itself or the inputs to the clock.

Future work

The discussion of the results obtained using 5-HTPacc to asses TrOH activity highlights the great gaps in knowledge of the intracellular mechanisms affecting the central 5-HT system. Below are listed several suggestions of work that could be initiated to further the understanding of the 5-HT system.

+ It would be very useful have a measure the number and affinity of presynaptic 5-HT_{1B} binding sites over 24 hours in all brain regions that receive a major 5-HT innervation. The numbers and affinity of somatodendritic and terminal heteroreceptors need to be assessed through the light-dark cycle. Further characterisation of these receptors and their effects on 5-HT synthesis and release over 24 hours in all brain regions would be very useful. Their anatomical location should be discovered to determine whether they are presynaptic heteroreceptors or not. The effect of chronic antidepressant treatment, using antidepressant drugs from differing classes, on putative heteroreceptor control of 5-HT synthesis and release should be investigated through the light-dark cycle. All the experiments above should be conducted in entrained animals as well as animals freely-running in constant dark and constant light.

+ The precise anatomical location of the 5-HT_{1B} receptor affecting 5-HT synthesis could be determined either by selective lesioning of different neurotransmitter systems or by inhibiting 5-HT_{1B} receptor expression in 5-HT neurons.

+ The effect of differing types of antidepressant drugs on basal TrOH activity needs to be fully addressed both *in vivo* and *in vitro,* with tissue being taken from treated animals.

+ The intracellular factors controlling TrOH activity ought to be elucidated. Similarly the regulation of 5-HT_{1B} receptor and TrOH expression needs to be deciphered. Measurement of the mRNA for 5-HT_{1B} receptors and TrOH, and the factors affecting their regulation needs to be assessed over 24 hours before and after chronic antidepressant treatment in specific brain regions.

+The adenylate cyclase type linked to 5-HT_{1B} receptors needs to be investigated in all brain regions. The activity of adenylate cyclase, its stimulation of PKA and the activity

of PKA all need to be measured over 24 hours before and after chronic antidepressant treatment.

Experiments, initially at mid and end dark, should be performed to complete the *in vivo* microdialysis study. The study could then be expanded to include antidepressant drugs from different classes and more time points.

More generally, the role presynaptic 5-HT_{1B} autoreceptors play in affecting circadian rhythms should be investigated in the SCN and IGL.

Circadian measurements of l-try levels in plasma, brain and 5-HT synthesis and release in depressed patients grouped according to the type of depression might help to provide more evidence for both a 5-HT involvement in different subtypes of depression including depression with a circadian element.

The results of these findings should help to elucidate the control the terminal 5-HT_{1B} autoreceptor exerts on the function of the 5-HT neuron, as well as helping to untangle how antidepressant drugs are effective in depression through the 5-HT system. Additionally it may help to understand how antidepressant drugs affect receptor rhythms and to what extent 5-HT is involved in depression with a circadian element.

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Appendix 1

Statistical methods

One-way or two-way analysis of variance was performed to indicate whether there were any differences between groups compared. However ANOVA does not identify where the significant differences lie. To determine differences tests based on Studentised range (Q) tables were performed. The test allows the comparison of any group with any other and is therefore conservative to keep the overall significance level.

A variance estimate, both values being obtained from the ANOVA, is calculated from the formula:

$$\text{variance estimate } (V_e) = \sqrt{\frac{\text{residual sum of squares}}{\text{residual degrees of freedom}}}$$

A critical value is then calculated using the formula:

(1) Tukey method for equal group sizes

$$\text{critical value} = \sqrt{\frac{V_e}{n} \times Q}$$

(2) Tukey/Kramer method for unequal group sizes

$$\text{critical value} = \sqrt{\frac{V_e \times (1/n_1 + 1/n_2)}{2} \times Q}$$

where n is the number observations per group and Q is derived from Studentised range (Q) tables from the residual degrees of freedom and the number of groups compared. Q values for $P<0.05$ and $P<0.01$ are obtained.

The differences between groups being compared are calculated and compared to the critical value. A difference greater than the critical value is considered significant at either the $P<0.05$ or $P<0.01$ level depending on the difference.

Statistical analysis and outcomes of 5-HTP accumulation experiments

Basal synthesis rate

The results of one-way ANOVA (time) on the variation in basal 5-HTP levels over 24 hours

	Hypothalamus	Hippocampus	Frontal Cortex	Striatum
df	3	3	3	3
F	28.8	40.5	29.5	116
P	1.8×10^{-7}	1.1×10^{-7}	1.5×10^{-7}	8.1×10^{-13}

Table A1. Accumulation of 5-HTP in four brain regions after decarboxylase inhibition. Values are expressed in ng 5-HTP/μg protein as mean±s.e.m., n=6 for each time point and brain region, differences in levels were determined by one-way ANOVA followed by Q test, $^{a}P<0.01$ mid light vs end dark, $^{b}P<0.05$ end light vs mid dark, $^{c}P<0.05$ end light vs end dark, $^{d}P<0.01$ mid dark vs end dark, $^{e}P<0.01$ mid light vs end light, $^{f}P<0.01$ mid light vs end dark, $^{g}P<0.05$ end light vs mid dark, $^{h}P<0.01$ mid dark vs end dark, $^{i}P<0.01$ mid light vs end dark, $^{j}P<0.01$ end light vs end dark, $^{k}P<0.01$ mid dark vs end dark, $^{l}P<0.01$ mid light vs end light, $^{m}P<0.01$ mid light vs end dark, $^{n}P<0.01$ end light vs mid dark, $^{o}P<0.01$ end light vs end dark and $^{p}P<0.01$ mid dark vs end dark.

Table A1. Variation in 5-hydroxytryptophan accumulation over 24 hours in four brain regions

	Time Point			
Brain Region	*Mid Light*	*End Light*	*Mid Dark*	*End Dark*
Hypothalamus	1.08 ± 0.06^{a}	0.94 ± 0.04^{bc}	1.15 ± 0.06^{bd}	0.75 ± 0.03^{acd}
Hippocampus	6.7 ± 0.3^{ef}	4.65 ± 0.2^{eg}	5.9 ± 0.4^{gh}	3.8 ± 0.2^{fh}
Frontal Cortex	7.0 ± 0.3^{i}	6.8 ± 0.45^{j}	6.3 ± 0.4^{k}	3.6 ± 0.2^{ijk}
Striatum	1.7 ± 0.09^{lm}	4.7 ± 0.4^{lno}	2.8 ± 0.2^{np}	7.4 ± 0.2^{mop}

Pharmacological Manipulation

Results of two-way ANOVA (treatment (trt) and time) on vehicle control animals from RU24969 group and RU24969 +/- (±)cyanopindolol.

		df	F	P
Hypothalamus	Trt	1	0.003	0.95
	Time	3	21.5	5.4×10^{-7}
	Trt x Time	3	0.002	0.99
Hippocampus	Trt	1	1.5	0.23
	Time	3	327	1.6×10^{-19}
	Trt x Time	3	3.48	0.03
Frontal Cortex	Trt	1	0.35	0.55
	Time	3	66.5	8.9×10^{-12}
	Trt x Time	3	0.37	0.77
Striatum	Trt	1	0.24	0.62
	Time	3	1368	5.9×10^{-27}
	Trt x Time	3	3.74	0.02

The results of two-way ANOVA (treatment (Trt) and time) on the effect of pharmacological agents (RU24969, (±)cyanopindolol (CP) and (+)WAY100135 (WAY)) on 5-HTP accumulation in the hypothalamus.

	RU24969			RU24969 +/- (±)CP			RU24969 +/- WAY		
	df	F	P	df	F	P	df	F	P
Trt	2	42.2	3.6×10^{-10}	2	4.7	0.015	2	18	1.1×10^{-5}
Time	3	36.1	6×10^{-11}	3	60	4.5×10^{-14}	3	13	0.0001
Trt x Time	6	0.32	0.91	6	8.3	1.2×10^{-5}	6	0.16	0.95

The results of two-way ANOVA (treatment (Trt) and time) on the effect of pharmacological agents (RU24969, (±)cyanopindolol (CP) and (+)WAY100135 (WAY)) on 5-HTP accumulation in the hippocampus.

	RU24969			RU24969 +/- (±)CP			RU24969 +/- WAY		
	df	F	P	df	F	P	df	F	P
Trt	2	348	2.8×10^{-24}	2	1.9	0.16	2	59	1.3×10^{-10}
Time	3	713	4.2×10^{-32}	3	47	1.5×10^{-12}	3	75	9.7 x 10
Trt x Time	6	88	5.5×10^{-20}	6	9.5	$3.x 10^{-6}$	6	5.1	0.003

The results of two-way ANOVA (treatment (Trt) and time) on the effect of pharmacological agents (RU24969, (±)cyanopindolol (CP) and (+)WAY100135 (WAY)) on 5-HTP accumulation in the frontal cortex.

	RU24969			RU24969 +/- (±)CP			RU24969 +/- WAY		
	df	F	P	df	F	P	df	F	P
Trt	2	53	2.1×10^{-11}	2	0.29	0.75	2	48	1.2×10^{-9}
Time	3	159	8.2×10^{-21}	3	77	9.8×10^{-16}	3	7.4	0.003
Trt x Time	6	3.3	0.011	6	1.7	0.14	6	1.8	0.16

The results of two-way ANOVA (treatment (Trt) and time) on the effect of pharmacological agents (RU24969, (±)cyanopindolol (CP) and (+)WAY100135 (WAY)) on 5-HTP accumulation in the striatum.

	RU24969			RU24969 +/- (±)CP			RU24969 +/- WAY		
	df	F	P	df	F	P	df	F	P
Trt	2	530	2.0×10^{-27}	2	3.4	0.04	2	14.2	6.0×10^{-5}
Time	3	8361	1.8×10^{-51}	3	235	1.0×10^{-23}	3	126	2.0×10^{-14}
Trt x Time	6	76	5.6×10^{-19}	6	3.8	0.004	6	0.93	0.46

Table A2. Effect of various manipulations on the accumulation of 5-HTP in the **hypothalamus** after decarboxylase inhibition. Values are expressed in ng 5-HTP/μg protein, as mean±s.e.m., n=4-8 depending on time point and treatment. For simplicity, values obtained from the control animals of each treatment group have been pooled and termed vehicle. Differences between treatments and time were determined by two-way ANOVA followed by Studentised range test, *P<0.05 vs pooled vehicle control **P<0.01 vs pooled vehicle control, ^{a}P<0.01 mid light vs mid dark, ^{b}P<0.01 mid light vs end dark. Data from experiments using WAY100135 have been normalised, see text on page 71 for details.

RU24969 vs group vehicle control P<0.001 with treatment, P<0.001 with time and Pns (P not significant) interaction; (±)Cyanopindolol + RU24969/ (±)cyanopindolol vs group vehicle control P<0.05 with treatment, P<0.001 with time and P<0.001 interaction; WAY100135 + RU24969/ WAY100135 vs group vehicle control P<0.001 with treatment, P<0.001 with time and Pns interaction.

Table A2. 5-Hydroxytryptophan accumulation in the hypothalamus

	Time Point			
Treatment	*Mid Light*	*End Light*	*Mid Dark*	*End Dark*
Vehicle	1.13±0.1	0.91±0.09	1.10±0.08	0.76±0.10
3mg/kg RU24969	1.08±0.05	0.87±0.02	1.14±0.05	0.73±0.06
9mg/kg RU24969	0.80±0.01**	0.66±0.03**	0.84±0.06**	0.52±0.07**
3mg/kg (±)Cyanopindolol + 9mg/kg RU24969	1.50±0.03**ab	0.90±0.01	1.21±0.05[a]	0.66±0.06[b]
3mg/kg (±)Cyanopindolol	1.13±0.1	1.00±0.03	1.07±0.03	0.80±0.02
5mg/kg WAY100135 + 9mg/kg RU24969	0.88±0.02**	0.67±0.01**	0.78±0.03**	0.58±0.04*
5mg/kg WAY100135	1.08±0.1	0.87±0.03	1.02±0.03	0.81±0.07

Table A3. Effect of various manipulations on the accumulation of 5-HTP in the **hippocampus** after decarboxylase inhibition. Values are expressed in ng 5-HTP/μg protein, as mean±s.e.m., n=4-8 depending on time point and treatment. For simplicity, values obtained from the control animals of each treatment group have been pooled and termed vehicle. Differences with time and treatment were determined by two-way ANOVA followed by Studentised range test, *P<0.05 vs vehicle, **P<0.01 vs vehicle, [a]<P0.01 mid light vs mid dark, [b]P<0.01 end light vs mid dark, [c]P<0.05 end light vs end dark, [d]P<0.05 mid dark vs end dark, [e]P<0.01 mid light vs end light, [f]P<0.01 mid light vs mid dark, [g]P<0.01 mid light vs end dark, [h]P<0.01 end light vs mid dark and [i]P<0.01mid dark vs end dark. Data from experiments using WAY100135 have been normalised, see text on page 71 for details.

RU24969 vs group vehicle control P<0.001 with treatment, P<0.001 with time and P<0.001 interaction; (±)Cyanopindolol + RU24969/ (±)cyanopindolol Pn.s. vs group vehicle control with treatment, P<0.001 with time and P<0.001 interaction; WAY100135 + RU24969/ WAY100135 vs group vehicle control P<0.001 with treatment, P<0.001 with time and P<0.01 interaction.

Table A3. 5-Hydroxytryptophan accumulation in the hippocampus

Treatment	Time Point			
	Mid Light	*End Light*	*Mid Dark*	*End Dark*
Vehicle	7.1±0.06	4.4±0.04	5.7±0.03	4.2±0.06
3mg/kg RU24969	7.3±0.02[a]	4.0±0.02*[bc]	4.2±0.1**[abcd]	4.2±0.2[cd]
9mg/kg RU24969	5.1±0.01**[efg]	3.7±0.1**[eh]	2.8±0.03**[fhi]	3.8±0.01**[gi]
3mg/kg (±)Cyanopindolol + 9mg/kg RU24969	7.3±0.6	4.3±0.1	5.4±0.3	4.2±0.2
3mg/kg (±)Cyanopindolol	6.2±0.3	5.7±0.2*	5.1±0.1	5.5±0.3**
5mg/kg WAY100135 + 9mg/kg RU24969	5.2±0.1**	3.5±0.2**	2.9±0.1**	3.6±0.1*
5mg/kg WAY100135	7.2±0.2	4.4±0.1	5.2±0.4	4.8±0.4

Table A4. Effect of various manipulations on the accumulation of 5-HTP in the **frontal cortex** after decarboxylase inhibition. Values are expressed in ng 5-HTP/μg protein, as mean±s.e.m., n=4-8 depending on time point and treatment. For simplicity, values obtained from the control animals of each treatment group have been pooled and termed vehicle. Differences with treatment and time were determined by two-way ANOVA followed by Studentised range test, *P<0.05 vs vehicle, **P<0.01 vs vehicle, ^{a}P<0.01 mid light vs end light ^{b}P<0.05 end light vs end dark, ^{c}P<0.01 end light vs mid dark, ^{d}P<0.01 end light vs end dark. Data from experiments using WAY100135 have been normalised, see text on page 71 for details.

RU24969 vs group vehicle control P<0.001 with treatment, P<0.001 with time and P<0.05 interaction; (±)Cyanopindolol + RU24969/ (±)cyanopindolol vs group vehicle control Pn.s. with treatment, P<0.001 with time and Pn.s. interaction; WAY100135 + RU24969/ WAY100135 vs group vehicle control P<0.001 with treatment, P<0.05 with time and Pn.s. interaction.

Table A4. 5-Hydroxytryptophan accumulation in the frontal cortex.

	Time Point			
Treatment	*Mid Light*	*End Light*	*Mid Dark*	*End Dark*
Vehicle	7.1±0.4	7.0±0.2	6.6±0.1	3.8±0.3
3mg/kg RU24969	7.0±0.4[a]	5.9±0.5**[ab]	7.2±0.1	3.6±0.04[b]
9mg/kg RU24969	5.6±0.2**	4.9±0.1**[cd]	5.6±0.2**[c]	2.9±0.1*[d]
3mg/kg (±)Cyanopindolol + 9mg/kg RU24969	6.4±0.4	7.1±0.4	6.1±0.2	3.9±0.1
3mg/kg (±)Cyanopindolol	6.7±0.4	6.5±0.2	6.0±0.1	4.1±0.1
5mg WAY100135 + 9mg/kg RU24969	5.9±0.1**	4.5±0.2**	4.9±0.2**	2.9±0.3*
5mg/kg WAY100135	7.4±0.6	5.7±0.2	6.9±0.1	3.8±0.1

Table A5. Effect of various manipulations on the accumulation of 5-HTP in the **striatum** after decarboxylase inhibition. Values are expressed in ng 5-HTP/μg protein, as mean±s.e.m., n=4-8 depending on time point and treatment. For simplicity, values obtained from the control animals of each treatment group have been pooled and termed vehicle. Differences with time and treatment were analysed by two-way ANOVA followed by Studentised range test *P<0.05 vs vehicle, **P<0.01 vs vehicle, ^{a}P<0.05 mid light vs end light, ^{b}P<0.05 mid light vs mid dark, ^{c}P<0.05 end light vs end dark, ^{d}P<0.05 mid dark vs end dark, ^{e}P<0.01 mid light vs end light, ^{f}P<0.01 mid light vs mid dark, ^{g}P<0.01 mid light vs end dark and ^{h}P<0.05 end light vs mid dark, ^{i}P<0.01 end light vs end dark, ^{j}P<0.01 mid dark vs end dark, ^{k}P<0.01 mid light vs end dark, ^{l}P<0.001 end light vs end dark, ^{m}P<0.001 mid dark vs end dark, ^{n}P<0.05 end light vs end dark. Data from experiments using WAY100135 have been normalised, see text on page 71 for details.

RU24969 vs group vehicle control P<0.001 with treatment, P<0.001 with time and P<0.001 interaction; (±)Cyanopindolol + RU24969/ (±)cyanopindolol vs group vehicle control P<0.001 with treatment, P<0.001 with time and Pn.s. interaction; WAY100135 + RU24969/ WAY100135 vs group vehicle control P<0.05 with treatment, P<0.001 with time and P<0.01 interaction.

Table A5. 5-Hydroxytryptophan accumulation in the striatum.

	Time Point			
Treatment	*Mid Light*	*End Light*	*Mid Dark*	*End Dark*
Vehicle	1.8±0.03	4.9±0.01	3.0±0.03	7.7±0.05
3mg/kg RU24969	1.6±0.06*ab	4.7±0.03ac	3.1±0.1bd	8.0±0.1cd
9mg/kg RU24969	1.5±0.06**efg	4.1±0.2**ehi	2.0±0.1**fhj	6.1±0.06**gij
3mg/kg (±)Cyanopindolol + 9mg/kg RU24969	1.9±0.1k	5.1±0.1l	3.1±0.1m	5.8±0.3**klm
3mg/kg (±)Cyanopindolol	1.8±0.1	4.6±0.5n	2.9±0.2	6.5±0.3*n
5mg/kg WAY100135 + 9mg/kg RU24969	1.6±0.08**	3.9±0.1**	1.9±0.1**	5.8±0.4**
5mg/kg WAY100135	1.8±0.1	4.6±0.5	2.9±0.2	7.4±0.3

Chronic antidepressant treatment

Results of one-way ANOVA (time) om 5-HTP accumulation after chronic antidepressant treatment at end light.

	Hypothalamus	Hippocampus	Frontal Cortex	Striatum
df	2	2	2	2
F	0.81	0.40	0.004	111.8
P	0.47	0.67	1.0	4.4×10^{-7}

Results of two-way ANOVA (treatment (trt) and time) on the effect of chronic antidepressant treatment on the RU24969-induced suppression of 5-HTP accumulation.

		df	F	P
Hypothalamus	Trt	2	0.96	0.39
	Time	3	41.8	8×10^{-12}
	Trt x Time	6	12.6	1.3×10^{-7}
Hippocampus	Trt	2	0.7	0.47
	Time	3	63.3	2×10^{-14}
	Trt x Time	6	6.0	0.0002
Frontal Cortex	Trt	2	33.1	6.98×10^{-9}
	Time	3	439	2.11×10^{-28}
	Trt x Time	6	37.6	4.52×10^{-14}
Striatum	Trt	2	10.3	0.0003
	Time	3	632	3.5×10^{-31}
	Trt x Time	6	20.6	2.6×10^{-10}

Table A6. Effect of chronic antidepressant treatment on the response to 9mg/kg i.p. RU24969 in the **hypothalamus**. Data is expressed in ng 5-HTP/μg protein, as mean±s.e.m., n=4 depending on each time point and treatment. Differences with treatment and time were determined by two-way ANOVA with post hoc Studentised range test, **P<0.01 vs saline-treated + 9mg/kg RU24969 ^{a}P<0.01 mid light vs end light, ^{b}P<0.01 mid light vs mid dark.

Basal 5-HTP levels were unaltered after chronic desipramine or paroxetine treatment ($F_{2,9}$=4.25, variance ratio 0.80). The effect of chronic antidepressant treatment significantly altered the response to RU24969 P<0.05 with treatment, P<0.001 with time and P<0.001 interaction.

Table A6. Effect of chronic antidepressant treatment on the response to RU24969 in the hypothalamus.

	Time Point			
Treatment	*Mid Light*	*End Light*	*Mid Dark*	*End Dark*
Saline + RU24969	0.72±0.05	0.69±0.03	0.92±0.01	0.6±0.01
Paroxetine + RU24969	0.69±0.03	0.80±0.04*	0.93±0.01	0.65±0.07
Desipramine + RU24969	1.02±0.02**ab	0.74±0.02a	0.90±0.03b	0.48±0.01**d
Saline		0.89±0.04		
Paroxetine		0.85±0.02		
Desipramine		0.83±0.04		

Table A7. Effect of chronic antidepressant treatment on the response to 9mg/kg i.p. RU24969 in the **hippocampus**. Values are expressed in ng 5-HTP/μg protein or †as a % of the saline value since analysis was not performed on the same HPLC system, as mean±s.e.m., n=4-6 depending on time point and treatment. Differences with treatment and time were determined by two-way ANOVA followed by Studentised range test, *$P<0.05$ compared to saline-treated + 9mg/kg RU24969, $^{a}P<0.01$ mid light vs end light, $^{b}P<0.01$ mid light vs mid dark, $^{c}P<0.01$ mid light vs end dark.

Basal 5-HTP levels were unaltered after chronic desipramine or paroxetine treatment ($F_{2,12}=3.88$, variance ratio 0.41). The effect of chronic antidepressant treatment significantly altered the response to RU24969 $P<0.05$ with treatment, $P<0.001$ with time and $P<0.001$ interaction.

Table A7. Effect of chronic antidepressant treatment on the response to RU24969 in the hippocampus.

	Time Point			
Treatment	*Mid Light*	*End Light*	*Mid Dark*	*End Dark*
Saline + RU24969	5.5±0.4	4.1±0.2	3.0±0.03	3.3±0.3
Paroxetine + RU24969	4.1±0.02**abc	4.4±0.02[a]	2.8±0.01[b]	3.8±0.02[c]
Desipramine + RU24969	5.1±0.02	3.9±0.02	2.9±0.01	3.6±0.1
Saline		100±4[†]		
Paroxetine		91±10[†]		
Desipramine		99±7[†]		

Table A8. Effect of chronic antidepressant treatment on the response to 9mg/kg i.p. RU24969 in the **frontal cortex**. Values are expressed in ng 5-HTP/μg protein or †as a % of the saline value since analysis was not performed on the same HPLC system, as mean±s.e.m., n=4-6 depending on time point and treatment. Differences with treatment and time were determined by two-way ANOVA followed by Studentised range test, **P<0.01 vs saline-treated + 9mg/kg RU24969, [a]P<0.01 mid light vs mid dark, [b]P<0.01 mid light vs end dark, [c]P<0.01 end light vs mid dark, [d]P<0.01 end light vs end dark, [e]P<0.01 mid light vs mid dark, [f]P<0.01 end light vs mid dark, [g]P<0.01 mid dark vs end dark.

Basal 5-HTP levels were unaltered after chronic desipramine or paroxetine treatment ($F_{2,12}$=3.88, variance ratio 0.41). The effect of chronic antidepressant treatment significantly altered the response to RU24969 P<0.05 with treatment, P<0.001 with time and P<0.001 interaction.

Table A8. Effect of chronic antidepressant treatment on the response to RU24969 in the frontal cortex.

	Time Point			
Treatment	*Mid Light*	*End Light*	*Mid Dark*	*End Dark*
Saline + RU24969	5.4±0.2	4.3±0.05	5.1±0.1	3.4±0.1
Paroxetine + RU24969	6.3±0.03**ab	5.0±0.01**c	5.3±0.01acd	3.5±0.04bd
Desipramine +RU24969	4.8±0.03**e	4.85±0.01**f	6.3±0.04**efg	3.1±0.02g
Saline		100±5†		
Paroxetine		101±3†		
Desipramine		93±8†		

Table A9. Effect of chronic antidepressant treatment on the response to 9mg/kg RU24969 in the **striatum.** Values are expressed in ng 5-HTP/μg protein or †as a % of the saline value since analysis was not performed on the same HPLC system, as mean±s.e.m., n=3-6. Differences with treatment and time were determined by two-way ANOVA followed by Studentised range test, *P<0.05 vs saline-treated + 9mg/kg RU24969, **P<0.01 vs untreated + 9mg/kg RU24969, ^{a}P<0.01 mid light vs end dark, ^{b}P<0.01 end light vs end dark, ^{c}P<0.01 mid dark vs end dark.

Basal 5-HTP levels were unaltered after chronic desipramine or paroxetine treatment compared to saline-treated animals ($F_{2,9}$=4.25, variance ratio 111.8, the significance was found to stem from a significant difference between 5-HTP levels in paroxetine-treated animals vs desipramine-treated animals.). The effect of chronic antidepressant treatment significantly altered the response to RU24969 P<0.001 with treatment, P<0.001 with time and P<0.001 interaction.

Table A9. Effect of chronic antidepressant treatment on the response to RU24969 in the striatum.

	Time Point			
Treatment	*Mid Light*	*End Light*	*Mid Dark*	*End Dark*
Saline + RU24969	1.7±0.08	4.0±0.1	2.1±0.2	6.8±0.4
Paroxetine + RU24969	1.1±0.04**	4.8±0.01**	2.5±0.01	6.4±0.07
Desipramine + RU24969	1.7±0.02[a]	4.5±0.01[b]	1.9±0.07[c]	4.8±0.05**abc
Saline		4.8±0.03		
Paroxetine		4.5±0.01		
Desipramine		4.9±0.02		

In vivo microdialysis

Control vs calcium free buffer:

P<0.001 with treatment, P<0.01 with time and P<0.001 interaction.

Control vs 1μM 8-OH-DPAT:

Pn.s. with treatment, P<0.05 with time and Pn.s. interaction.

Control vs 0.1μM RU24969:

P<0.001 with treatment, P<0.01 with time and P<0.01 interaction.

Control vs 1μM RU24969:

P<0.001 with treatment, Pn.s. with time and P<0.01 interaction.

Control vs 5μM RU24969:

P<0.001 with treatment, Pn.s. with time and P<0.05 interaction.

Control vs 10μM RU24969:

P<0.001 with treatment, P<n.s. with time and P<0.001 interaction.

Control vs 1μM methiothepin:

P<0.001 with treatment, P0.01 with time and P<n.s.

Control vs 10μM methiothepin:

P<0.001 with treatment, P<0.01 with time and P n.s. interaction.

1μM RU24969 vs 1μM methiothepin + 1μM RU24969:

P<0.001 with treatment, Pn.s. with time and P<0.05 interaction.

5μM RU24969 vs 10μM methiothepin + 5μM RU24969:

P<0.001 with treatment, Pn.s. with time and Pn.s. interaction.

Mid light control vs 5μM RU24969:

P<0.001 with treatment, P<0.01 with time and P<0.001 interaction.

End light vs 5μM RU24969:

P<0.001 with treatment, P<0.001 with time and P<0.001 interaction.

Mid light saline-treated vs antidepressant-treated:

P<0.001 with treatment, P<0.001 with time and P<0.05 interaction.

End light saline-treated vs antidepressant-treated:

P<0.001 with treatment, P<0.001 with time and Pn.s. interaction.

Table A10. Results of *in vivo* microdialysis experiments. Values are expressed in % control values as mean±s.e.m., for n see graphs on pages 128.

	Time (mins)								
Treatment	*-30*	*-15*	*0*	*15*	*30*	*45*	*60*	*75*	*90*
Pooled control	101±0.2	99±0.2	96±0.4	102±0.8	84±0.3	85±0.7	85±0.5	86±0.5	72±0.4
Ca^{++} free	105±0.7	95±0.7	63±3.7	11±3.7	5±1.2	2±0.6	9.5±1.4	70±1.9	55±2.7
1μM 8-OH-DPAT	101±0.8	99±0.8	96±1.2	103±2.5	98±1.2	92±1.3	95±2.4	105±4.6	76±0.7
0.1μM RU24969	102±2.4	98±2.4	77±9.7	46±3.3	45±6.6	58±3.0	67±4.6	60±3.6	92±7.4
1μM RU24969	104±1.0	96±1.0	61±2.0	61±2.0	47±4.0	53±3.0	53±2.0	63±0.8	66±2.0
5μM RU24969	101±1.7	99±1.7	66±1.0	59±1.9	48±3.0	44±1.7	49±2.2	61±5.2	37±2.2
10μM RU24969	102±0.9	98±0.2	81±2.0	60±2.0	35±2.0	41±4.0	49±3.0	58±2.0	65±1.0
1μM Methiothepin	98±1.8	102±1.8	110±3.6	116±5.2	113±4.2	115±5.8	113±3.4	107±3.4	99±1.9
10μM Methiothepin	100±0.8	100±0.8	95±2.1	149±11	238±8.3	221±9.8	242±7.1	153±7.4	130±1.5
1μM Methiothepin + 1μM RU24969	99±3.1	101±3.1	114±2.6	76±7.0	73±7.0	87±4.0	108±6.0	107±7.0	75±2.0

	Time (mins)								
Treatment	*-30*	*-15*	*0*	*15*	*30*	*45*	*60*	*75*	*90*
10μM Methiothepin + 5μM RU24969	102±0.2	98±0.2	102±4.6	115±6.4	83±3.8	131±6.7	122±6.0	122±7.8	137±6.8
Mid light control	101±0.8	99±0.8	97±4.4	100±1.7	92±1.5	97±1.3	99±1.7	88±1.5	84±1.4
5μM RU24969	100±0.9	100±0.9	87±2.8	58±2.3	48±1.1	35±3.3	37±2.7	39±0.4	88±3.2
Saline-treated + 5μM RU24969	97±0.5	103±0.5	79±1.7	70±1.0	65±1.7	61±2.9	54±2.9	89±4.3	129±3.8
Paroxetine-treated + 5μM RU24969	103±0.3	97±0.3	81±1.7	71±1.5	62±1.3	53±1.7	53±2.6	98±4.0	131±5.8
Desipramine-treated + 5μM RU24969	102±1.9	98±1.9	71±1.6	52±1.4	54±1.7	55±1.5	49±1.7	55±1.4	67±2.9

	Time (mins)								
Treatment	*-30*	*-15*	*0*	*15*	*30*	*45*	*60*	*75*	*90*
End light control	102±0.5	98±0.5	98±1.2	104±1.2	92±1.2	98±1.2	93±1.0	79±1.5	82±1.5
5μM RU24969	101±0.7	99±0.7	84±1.4	60±1.0	36±3	9±2.2	0±0.0	0±0.0	0±0.0
Saline-treated + 5μM RU24969	101±0.7	99±0.7	80±2.0	69±3.9	57±2.5	40±1.9	28±2.6	1.2±1.2	1.2±1.2
Paroxetine-treated + 5μM RU24969	98±0.2	102±0.2	69±2.4	65±2.0	71±3.1	86±3.1	88±3.1	92±0.5	92±0.8
Desipramine-treated + 5μM RU24969	102±0.8	98±0.8	82±6.7	72±7.4	53±5.0	30±1.8	17±0.9	7±0.8	8±0.9